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(54) **SAPONIN-DIGESTING ENZYMES, GENES THEREOF AND SOYASAPOGENOL B MASS PRODUCTION SYSTEM**

SAPONIN VERDAUENDE ENZYME, GENE DAFÜR SOWIE EIN SOYASAPOGENOL-B-MASSENPRODUKTIONSSYSTEM

ENZYMES DIGERANT LA SAPONINE, GENES DE CELLES-CI ET SYSTEME DE PRODUCTION EN MASSE DE SOYASAPOGENOL B

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Description

[BACKGROUND OF THE INVENTION]

Field of the Invention

[0001] The present invention relates to a novel saponin-decomposing enzyme, a gene thereof, and a novel method for producing soyasapogenol B using them.

Background Art

[0002] Soyasapogenol B (12-oleanane-3,22,24-triol) is one of the aglycones of saponins contained in legumes and has been reported to have various physiological activities since early times. For example, platelet aggregation suppressing effect, anticomplementary activity, and preventive and therapeutic activity for nephritis, rheumatism, immune diseases such as systemic lupus erythematosus, autoimmune diseases or thrombosis have been reported (Chem. Pharm. Bull., 24, 121-129, 1976; Chem. Pharm. Bull., 30, 2294-2297, 1982; Kagaku to Seibutsu, 21, 224-232, 1983; Japanese Patent Application Laid-open No. 37749/1986). Further, growth-suppressing effect on cells derived from human colon cancer and human ovarian cancer has been reported (Japanese Patent Application Laid-open No. 37749/1986; Japanese Patent Application Laid-open No. 234396/1998).

[0003] Soyasapogenol B can be produced, for example, by chemically hydrolyzing sugar chains of saponins contained in soybean seeds as glycosides (soyasaponins I-V). However, this is not an effective production method because a considerable number of by-products may be produced depending on the conditions for acid hydrolysis. Further, soybean seeds are known also to contain saponins which have soyasapogenol A (soyasaponins A1-A6) or soyasapogenol E as an aglycone. Therefore, when soyasapogenol B is prepared from soybeans, the resulting preparation may easily contain soyasapogenol A and soyasapogenol E as impurities so that it is difficult to purify soyasapogenol B alone from such preparation. Further, since the saponin content of soybean seeds is generally as low as about 0.2% (Yakugaku Zasshi, 104, 162-168, 1984), there is a need for more efficient production.

[0004] As for methods of producing soyasapogenol B using microorganisms, a method with genus Streptomyces (Chem. Pharm. Bull. 32: 1287-1293, 1984) and a method with genus Penicillium (Japanese Patent Application Laid-open No. 234396/1998) is known. However, these methods of producing soyasapogenol B using microorganisms are poor in productivity and practicality.

[0005] Further, it has been reported that soyasapogenol B can be obtained as a by-product in a method in which an acid oligosaccharide having glucuronic acid as the reduced end is produced by hydrolyzing a glucuronide saponin using the enzyme (glucuronidase) produced by microorganisms that belong to genus Aspergillus or a culture containing this enzyme (Japanese Patent Publication No. 32714/1995). However, this method is primarily a method of producing acid oligosuccharides, and only a qualitative confirmation of soyasapogenol B is described in this report. Further, this report revealed the molecular weight of the enzyme having activity of interest but not the amino acid sequence thereof.

[0006] On the other hand, the search for microorganisms which efficiently produce soyasapogenol B by selectively hydrolyzing a glycoside having soyasapogenol B as an aglycone resulted in finding filamentous fungus strains that belong to genus Neocosmospora or genus Eupenicillium. It has been found that soyasapogenol B is produced and accumulated in a culture medium at a high concentration by culturing filamentous fungi, that belong to genus Neocosmospora or genus Eupenicillium, in a medium containing a saponin (a glycoside having soyasapogenol B as an aglycone) (see WO 01/81612) .

[0007] Examples of such filamentous fungi include Neocosmospora vasinfecta var. vasinfecta PF1225 that belongs to genus Neocosmospora and Eupenicillium brefeldianum PF1226 that belongs to genus Eupenicillium (see WO 01/81612).

[0008] Soyasapogenol B of interest can be produced using such fungi as they are, depending on the amount of saponins added to the medium. However, the amount of saponins to be added to the medium is limited because of the surface-active property of saponins, which easily foam. Further, viscosity of the medium supplemented with saponins is expected to increase because of the surface-active property. Accordingly, in order to improve the yield in producing the target substance from the culture, the extraction process has to be repeated several times. Further, soybean extract, which is generally used as a natural resource to effectively supply saponins, usually contains components other than saponins, such as lipids, proteins and polysaccharides. Therefore, the possible amount of saponins to be added to a medium ultimately depends on the purity of the soybean extract, which does not necessarily assure efficient production.

[0009] There is a need to develop a method for the large scale production of soyasapogenol B by an enzyme reaction using a saponin-decomposing enzyme producing enzyme, in which soyasapogenol B is efficiently produced and a high yield is maintained independently of the saponin content in a soybean extract, contrary to conventional methods.

[0010] By way of further prior art, WO 95/30009 discloses polypeptides with saponin glycosyl hydrolase activity,

including avenacinase and tomatinase. The polypeptides may be used in deglycosylating saponins.

[SUMMARY OF THE INVENTION]

[0011] Recently, the present inventors succeeded in isolating and purifying a protein having saponin-decomposing activity from microorganisms having saponin-decomposing activity (occasionally called "saponin-decomposing enzyme" hereinafter) and in identifying a gene encoding this protein. Further, the present inventors were able to obtain a highly active saponin-decomposing enzyme by expressing the resulting gene in a heterologous host. Further, the present inventors were able to effectively produce soyasapogenol B by carrying out an enzyme reaction using the saponin-decomposing enzyme thus obtained. The present invention is based on these findings.

[0012] Accordingly, an objective of the present invention is to provide a protein having saponin-decomposing activity, more specifically a protein which can decompose a glycoside having soyasapogenol B as an aglycone to produce soyasapogenol B, a polynucleotide encoding such protein, and a method of producing soyasapogenol B on a large scale using the same.

[0013] A protein according to the present invention is selected from the group consisting of the followings:

(a) a protein comprising an amino acid sequence selected from the group consisting of the amino acid sequences shown in SEQ ID NOs: 2, 4, and 6; and
(b) a protein that has at least 95% homology to the protein comprising the amino acid sequence of the sequence described in (a) and having saponin-decomposing activity .

[0014] A polynucleotide according to the present invention is selected from the group consisting of the followings:

(i) a polynucleotide consisting of a DNA sequence selected from the group consisting of the DNA sequences of SEQ ID NOs: 1, 3, and 5;
(ii) a polynucleotide that has at least 95% homology to the polynucleotide consisting of the DNA sequence of (i) and encodes a protein having saponin-decomposing activity.

[0015] A recombinant vector according to the present invention comprises a polynucleotide of the present invention.

[0016] Further, a host according to the present invention is a host transformed with the abovementioned recombinant vector.

[0017] A process for producing a protein of interest according to the present invention comprises culturing the above-mentioned transformed host and collecting a protein having saponin-decomposing activity from the resulting culture.

[0018] According to the present invention, a highly active saponin-decomposing enzyme can be obtained. Further, by using this enzyme, soyasapogenol B can be obtained efficiently and on a large scale from saponin. According to this method, soyasapogenol B can be obtained independently of the saponin content of, for example, a soybean extract.

[BRIEF DESCRIPTION OF THE DRAWINGS]

[0019]

Figure 1 shows the construction and restriction map for plasmid pCB-SEe.
Figure 2 shows the optimum pH for recombinant saponin-decomposing enzymes in Example 5. In the Figure, the wild-type SDN means the saponin-decomposing enzyme derived from Neocosmospora vasinfecta var. vasinfecta PF1225, and the recombinant SDN means the recombinant saponin-decomposing enzyme.
Figure 3 shows the optimum temperature for recombinant saponin-decomposing enzymes in Example 5. In the Figure, the wild-type SDN means the saponin-decomposing enzyme derived from Neocosmospora vasinfecta var. vasinfecta PF1225, and the recombinant SDN means the recombinant saponin-decomposing enzyme.
Figure 4 shows the construction and restriction map for plasmid pCB-SDAe.
Figure 5 shows the optimum pH for recombinant saponin-decomposing enzymes in Example 8. In the Figure, the wild-type SDA means the saponin-decomposing enzyme derived from Aspergillus sp. PF1224, and the recombinant SDA means the recombinant saponin-decomposing enzyme.
Figure 6 shows the optimum temperature for recombinant saponin-decomposing enzymes in Example 8. In the Figure, the wild-type SDA means the saponin-decomposing enzyme derived from Aspergillus sp. PF1224, and the recombinant SDA means the recombinant saponin-decomposing enzyme.
Figure 7 shows the construction and restriction map for plasmid pCB-SDEs.
Figure 8 shows the optimum pH for recombinant saponin-decomposing enzymes in Example 12. In the Figure, the wild-type SDE means the saponin-decomposing enzyme derived from Eupenicillium brefeldianum PF1226, and the

recombinant SDN means the recombinant saponin-decomposing enzyme.

Figure 9 shows the optimum temperature for recombinant saponin-decomposing enzymes in Example 12. In the Figure, the wild-type SDE means the saponin-decomposing enzyme derived from Eupenicillium brefeldianum PF1226, and the recombinant SDE means the recombinant saponin-decomposing enzyme.

Figure 10 shows the result made a comparison with SDN, SDA and SDE by seaching their homology each other.

[DETAILED DESCRIPTION OF THE INVENTION]

Deposition of microorganisms

[0020] The strain Neocosmospora vasinfecta var. vasinfecta PF1225 was deposited with the International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, AIST Tsukuba Central 6, 1-1-1-Higashi, Tsukuba, Ibaraki, Japan 305-5466, dated March 13, 2000 (original deposition date). The accession number is FERM BP-7475.

[0021] The strain Eupenicillium brefeldianum PF1226 was deposited with the International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, AIST Tsukuba Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki, Japan 305-5466, dated March 13, 2000 (original deposition date). The accession number is FERM BP-7476.

[0022] The strain Aspergillus sp. PF1224 was deposited with the International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, AIST Tsukuba Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki, Japan 305-5466, dated May 24, 2001. The accession number is FERM BP-8004.

[0023] The strain Trichoderma viride MC300-1 was deposited with the International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, AIST Tsukuba Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki, Japan 305-5466, dated September 9, 1996 (original deposition date). The accession number is FERM BP-6047.

Protein having saponin-decomposing activity

[0024] A saponin-decomposing enzyme isolated and purified from Neocosmospora vasinfecta var. vasinfecta PF1225 (FERM BP-7475) was revealed to be a novel enzyme system since it has no homology to any saponin-decomposing enzyme found to date and is different from glucuronidase derived from a microorganism belonging to genus Aspergillus (a glycoprotein having a molecular weight of about 158,000 consisting of subunits each having a molecular weight of 35,000 and 45,000 described in Japanese Patent Publication No. 32714/1995), which decomposes glucuronide saponin, in its subunit structure and molecular weight.

[0025] Further the protein according to the present invention and glucuronidase previously disclosed in the Patent Publication were studied for their identity and homology. Since the strain belonging to genus Aspergillus described in said Patent Publication was not readily available, another strain belonging to genus Aspergillus was used. The strain used was Aspergillus sp. PF1224, which was identified to be a filamentous fungus, Deuteromycetes, belonging to genus Aspergillus according to the microbial properties shown below.

(1) Colony features

[0026] Colonies grow well on a Czapek's yeast extract agar medium at 25°C attaining a diameter of 80 mm in 7 days. The colonies are yellow to yellowish green, woolly and rich in conidia and sclerotia. The reverse side becomes pale brown. Colonies grow well on a malt extract agar medium at 25°C attaining a diameter of 80 mm in 7 days. The colonies are yellow to yellowish green, woolly and rich in conidia and sclerotia. The reverse side becomes ocherous. Colonies are slightly suppressed on either medium when cultured at 37°C.

(2) Morphological features

[0027] Conidial heads are yellow to yellowish green and radiate to loose cylindrical. Conidiophores are rough and colorless and vesicles are rodlike to subglobose, bearing aspergilla on almost the entire surface. Monoseriate and biseriate aspergilla are mixed; they are generally biseriate. Metulae are 8-12 $\times$ 4-5 μm and phialides are 8-12 $\times$ 3-4 μm. Conidia are globose to subglobose, rough, and 4-6 μm in length.

[0028] The enzyme referred to as glucuronidase was confirmed using Aspergillus sp. PF1224, which revealed that the saponin-decomposing enzyme isolated and purified from Aspergillus sp. PF1224 by the present inventors has a molecular weight of 90 kDa, an optimum pH of 5 to 6, and an optimum temperature of 45°C to 50°C (see Reference Example). Furthermore, it was revealed that the saponin-decomposing enzyme isolated and purified from Eupenicillium brefeldianum PF1226 has a molecular weight of 90 kDa, an optimum pH of 5 to 6, and an optimum temperature of 40°C to 45°C

[0029] The protein of the present invention and the glucuronidase described in the abovementioned Patent Publication were compared for their molecular weight and subunit configuration since information such as an amino acid sequence of glucronidase was not disclosed in this Patent Publication to compare homology of the amino acid sequence or the like. Results showed that the protein according to present invention is a protein different from the glucronidase described in the Patent Publication.

[0030] As mentioned above, the present invention provides a protein selected from the group consisting of:

(a) a protein comprising an amino acid sequence selected from the group consisting of the amino acid sequences shown in SEQ ID NOs: 2, 4, and 6; and

(b) a protein that has at least 95% homology to the protein comprising the amino acid sequence of the sequence described in (a) and having saponin-decomposing activity.

[0031] Namely, a protein according to the present invention comprises an amino acid sequence which is identical or substantially identical to the amino acid sequence shown in SEQ ID NO: 2, 4, or 6.

[0032] An amino acid sequence that is substantially identical to the amino acid sequence shown in SEQ ID NO: 2, 4, or 6 herein means an amino acid that is more than 95%, or preferably more than 98% homologous to any one of the amino acid sequences shown in these SEQ ID NOs.

[0033] Further, these figures for homology shown in the present specification can be any figures calculated using a homology search program known to the skilled in the art. For example, figures can be readily calculated using default parameters in FASTA, BLAST, or the like.

[0034] For example, when the figures for homology has been calculated using default parameters in the homology search program Genetyx (manufactured by Genetyx Co.), the figure for homology between SDN and SDA is 51%, that between SDA and SDE is 52%, and that between SDE and SDN is 51%.

[0035] In a more preferred embodiment of the invention, the protein described in (b) above is a protein comprising a modified amino acid sequence that has one or more conservatively substituted amino acid residues in the amino acid sequence (a) above and having saponin-decomposing activity.

[0036] The expression "conservatively substituted" herein means that one or more amino acid residues are substituted with other amino acid residues which are chemically homologous not to substantially alter the protein activity. For example, a hydrophobic residue is substituted with another hydrophobic residue or a polar residue is substituted with another polar residue having the same electric charge. Functionally homologous amino acids of different types, which can be conservatively substituted in this way, are known to the skilled in the art. Examples of such amino acids include non-polar (hydrophobic) amino acids such as alanine, valine, isoleucine, leucine, proline, tryptophan, phenyalanine, and methionine; polar (neutral) amino acids such as glycine, serine, threonine, tyrosine, glutamine, asparagine, and cysteine; positively charged (basic) amino acids such as arginine, histidine, and lysine; and further, negatively charged (acidic) amino acids such as aspartic acid, and glutamic acid.

[0037] In the present invention, the term "protein having saponin-decomposing activity" means a protein that is verified to have an activity to decompose saponin. For example it means a protein which is verified to have saponin-decomposing activity when measured under the same conditions as described in Example 5. This protein can be isolated and purified from "organisms having saponin-decomposing activity" described below.

[0038] A protein according to the present invention can be obtained, for example, as follows.

[0039] An organisms having saponin-decomposing activity is cultured and a protein having saponin-decomposing activity is isolated and purified from the resulting culture using the saponin-decomposing activity as an index. The amino acid sequence of the protein thus purified is analyzed, an oligonucleotide encoding this sequence is synthesized, and the PCR (polymerase chain reaction) is carried out using DNA of said organism as a template to synthesize a long probe. A DNA sequence of the translation region of the saponin-decomposing enzyme gene is analyzed by the inverse PCR or the RACE (rapid amplification of cDNA ends) method using this probe. The translation region of the saponin-decomposing enzyme thus obtained is linked to a regulatory sequence which functions in a host to be used for expression to obtain an expression vector. This expression vector is used to transform the host, the resulting transformant is cultured, and thus a saponin-decomposing enzyme can be obtained.

[0040] An "organism having saponin-decomposing activity" herein can be any organism having saponin-decomposing activity and is not particularly limited and includes microorganisms and plants. Examples of such microorganism include filamentous fungi that belong to genus Neocosmospora, genus Aspergillus, and genus Eupenicillium. These fungi are used in manufacturing soy sauce and soybean paste by fermentation and known to have saponin-decomposing activity. Further, said microorganisms include actinomycetes and bacteria since some of them may also have saponin-decomposing activity. Said plants include plant itself, plant cells, callus or culture cells derived from leguminous plants since some saponin glycotransferase of such plants may catalyze reverse reaction.

[0041] In a preferred embodiment of the present invention, a protein or a polynucleotide according to the present invention is derived from a microorganism, more preferably a microorganism belonging to filamentous fungus. A micro-

organism belonging to such filamentous fungus is preferably filamentous fungus belonging to genus Neocosmospora, genus Aspergillus, or genus Eupenicillium.

[0042] Examples of filamentous fungi that belong to genus Neocosmospora include Neocosmospora vasinfecta var. vasinfecta PF1225 (accession number: FERM BP-7475) and mutants thereof. Examples of filamentous fungi that belong to genus Aspergillus include Aspergillus sp. PF1224 (accession number: FERM BP-8004) and mutants thereof. Examples of filamentous fungi that belong to genus Eupenicillium include Eupenicillium brefeldianum PF1226 (accession number: FERM BP-7476) and mutants thereof.

Polynucleotide

[0043] The present invention provides a polynucleotide which encodes a protein of the present invention.

[0044] A polynucleotide according to the present invention is typically a polynucleotide selected from the group consisting of (i) to (ii) described above.

[0045] Namely, according to one embodiment of the present invention, the polynucleotide comprises a DNA sequence selected from the group consisting of the DNA sequences shown in SEQ ID NOs: 1, 3, and 5.

[0046] According to another embodiment of the present invention, the polynucleotide comprises a DNA sequence having at least 95% homology to the polynucleotide comprising the DNA sequence shown in SEQ ID NO: 1, 3, or 5 and encodes a protein having saponin-decomposing activity. The homology to the polynucleotide comprising the DNA sequence shown in SEQ ID NO: 1, 3, or 5 is preferably more than 98%.

[0047] Further, figures for homology shown in the present specification can be any figures calculated using a homology search program known to the skilled in the art. For example, figures can be readily calculated using default parameters in FASTA, BLAST, or the like.

[0048] Further, according to the present invention, the polynucleotide also implies a polynucleotide which is complementary to a polynucleotide encoding a protein having saponin-decomposing activity.

Recombinant vector

[0049] The present invention provides a recombinant vector comprising the abovementioned polynucleotide.

[0050] The procedure and method for constructing a recombinant vector according to the present invention can be any of those commonly used in the field of genetic engineering.

[0051] Examples of the expression vector as used herein include vectors which can be incorporated into a host chromosome DNA and vectors having a self-replicable autonomous replication sequence which can be present as a plasmid in a host cell, for example, pUC vectors (e.g., pUC18 and pUC118), pBluescript vectors (e.g., pBluescript II KS+), and plasmids such as pBR322 plasmid. One or more of copies of the gene can be present in a host cell.

[0052] A regulatory sequence for the recombinant vector can be any regulatory sequence which can function in a host and is not particularly limited. For example, a promoter, a terminator, and the like can be used. Such a regulatory sequence can be ligated to a gene encoding a protein having saponin-decomposing activity for the gene expression.

[0053] The ligation to a regulatory sequence can be carried out, for example, according to an ordinary method by inserting a translation region of a gene encoding a protein of interest (gene of interest) downstream of a promoter in the right direction. In this case, the protein can be expressed as a fusion protein by ligating the gene of interest to a foreign gene encoding a translation region of another protein.

[0054] The expressed protein having saponin-decomposing activity or the expressed fused protein having said activity can be produced in a host cell used for expression or released into a medium.

[0055] For example, a saponin-decomposing enzyme derived from Neocosmospora vasinfecta var. vasinfecta PF1225 (FERM BP-7475) was revealed to have a signal peptide sequence of 26 amino acid residues at the N-terminal side according to the DNA sequence analysis and N-terminal amino acid sequence analysis (see Example). Similarly, a saponin-dacomposing enzyme derived from Aspergillus sp. PF1224 and a saponin-decomposing enzyme derived from Eupenicillium brefeldianum PF1226 were revealed to have signal peptide sequences of 28 amino acid residues and 17 amino acid residues at the N-terminal side, respectively.

[0056] Accordingly, for example, when filamentous fungi such as those belonging to genus Trichoderma and genus Aspergillus are used as a host, the protein can be released into a medium by utilizing a signal sequence included in this sequence.

[0057] Further, the saponin-decomposing enzyme derived from Neocosmospora vasinfecta var. vasinfecta PF1225 having a molecular weight of about 77 kDa is inferred to be glycoproteins from a molecular weight of about 68 kDa estimated from a deduced amino acid composition and a molecular weight of about 68 kDa of a protein expressed in strains of Escherichia coli and Trichoderma viride. Similarly, the saponin-decomposing enzyme derived from Aspergillus sp. PF1224 having a molecular weight of about 90 kDa is inferred to be glycoproteins from a molecular weight of about 65 kDa estimated from a deduced amino acid composition and a molecular weight of about 80 kDa of a protein expressed

in strains of Trichoderma viride. Further, the saponin-decomposing enzyme derived from Eupenicillium brefeldianum PF1226 having a molecular weight of about 90 kDa is inferred to be glycoproteins from a molecular weight of about 65 kDa estimated from a deduced amino acid composition.

[0058] These sugar chain are presumed not to have great influence on the expression of activity. However, various modification after translation, such as addition of various sugar chains, can be carried out anticipating effective changes in heat resistance, optimum pH, stability during storage, or the like.

[0059] A recombinant vector according to the present invention can be constructed by further ligating a selective marker gene such as a drug resistance gene and/or a gene complementing a nutritional requirement.

[0060] A gene marker can be appropriately selected depending on the technique for selecting a transformant. For example, a gene encoding drug resistance or a gene complementing a nutritional requirement can be used. Examples of the drug resistance gene include genes conferring resistance to destomycin, benomyl, oligomycin, hygromycin, G418, pleomycin, bialaphos, blastcidin S, phleomycin, phosphinothricin, ampicillin, streptomycin, and kanamycin. Examples of the gene complementing a nutritional requirement include amds, pyrG, argB, trpC, niaD, TRP1, LEU2, and URA3. Further, a gene marker can be a gene complementing a nutrient requirement indigenous to a host to be used for expression in systems for synthesizing various amino acids, vitamins, nucleic acids, or the like, or a gene complementing a nutrient requirement that is rendered by various mutagenic treatments.

Production of transformant and protein of interest

[0061] The present invention provides a host transformed with the abovementioned recombinant vector.

[0062] A host to be used in the present invention is not particularly restricted and any organism which can properly transcript and translate a gene encoding a protein having saponin-decomposing activity can be used. Examples of the host include bacteria such as Escherichia coli and Bacillus spp., actinomycetes, yeasts, filamentous fungi such as Trichoderma spp. and mutants thereof.

[0063] A recombinant vector for the gene expression can be introduced into a host by an ordinary method. Examples of the method for the introduction include the electroporation method, the polyethylene glycol method, the aglobacterium method, the lithium method, and the calcium chloride method. A method effective to each host cell can be selected.

[0064] A transformant (transformed host cell) can be cultured according to an ordinary method by appropriately selecting a medium, culture conditions and the like.

[0065] Conventional components can be used in a medium. As a carbon source, glucose, sucrose, cellulose, starch syrup, dextrin, starch, glycerol, molasses, animal and vegetable oils, and the like can be used. As a nitrogen source, soybean powder, wheat germ, cornsteep liquor, cotton seed lees, bouillon, peptone, yeast extract, ammonium sulfate, potassium nitrate, urea, and the like can be used. If necessary, sodium, potassium, calcium, magnesium, cobalt, chlorine, phosphoric acid, sulfuric acid, and other inorganic salts that can produce ions, such as potassium chloride, magnesium sulfate, monopotassium phosphate, zinc sulfate, manganese sulfate, and copper sulfate, can be effectively added. If necessary, various vitamins, amino acids, trace nutrients such as nucleotides, and selective drugs such as antibiotics can be added. Further, organic and inorganic substances to promote the growth of transformants and enhance the expression of an introduced gene can be appropriately added.

[0066] Cultivation can be carried out in a medium selectively containing these components.

[0067] For example, in a liquid medium, the cultivation can be carried out using a culture method under an aerobic condition, a shaking culture method, an agitation culture method with aeration, a submerged culture method or the like. The pH of the medium is, for example, about 5 to 8. The cultivation can be carried out at a normal temperature, such as 14°C to 40°C, preferably 26°C to 37°C, for about 1 to 25 days.

[0068] In a method of producing a protein of interest according to the present invention, a gene expression product, namely the protein of interest having saponin-decomposing activity, can be obtained from the culture of transformed cells. The protein of interest can be obtained from the culture according to an ordinary method. For example, steps of the extraction from the culture (e.g., by mashing, and crushing under pressure), the recovery (e.g., by filtration and centrifugation), and/or the purification (e.g., by salting out and solvent precipitation) can be appropriately combined. Furthermore, in these steps, a protease inhibitor, such as phenylmethylsulfonyl fluoride (PMSF), benzamidine and leupeptin, can be added if necessary.

[0069] According to another embodiment of the present invention, it is also possible to express a gene encoding a protein having saponin-decomposing activity in a plant which produces saponins, such as plants of soybean, kidney bean, cowpea, pea, peanut, and broad bean, and alfalfa to generate a plant body containing soyasapogenol B, from which soyasapogenol B is directly obtained. In this case, actin, ubiquitin, cauliflower mosaic virus 35S promoter, or the like, or a regulatory sequence of a gene specifically expressed at a part, such as the seed, can be used.

[0070] A gene encoding a protein having saponin-decomposing activity is properly linked to such a regulatory sequence and further, a drug resistance gene conferring resistance to bialaphos, kanamycin, blastcidin S, or the like is linked if necessary. The resultant product can be introduced into a plant cell, for example, by a direct introduction method such

as the particle-gun method, the PEG method, the electroporation method, and the microinjection method, or by an indirect introduction method using Ti plasmid vector of aglobacterium to generate a transformed plant cell. Introduction of the gene into a plant cell or plant body can be carried out according the method of Vaeck M. et al (Nature, 328, 33-37, 1987).

[0071] The plant cell thus transformed can be redifferentiated by the method known to the skilled in the art into a complete body of a transformed plant. Further, the transformed plant is cultivated and the resultant whole plants and/or organs, such as seeds, in which a gene encoding a protein having saponin-decomposing activity is expressed are harvested, from which soyasapogenol B can be obtained using a method suitable for its property, such as the solvent extraction method.

Production of soyasapogenol B

[0072] Another embodiment of the present invention provides a method of producing soyasapogenol B which comprises culturing the abovementioned transformed host to produce a protein having saponin-decomposing activity, followed by using the protein to decompose a glycoside having soyasapogenol B as an aglycone.

[0073] Further, still another embodiment of the present invention provides a method of producing soyasapogenol B which comprises decomposing a glycoside having soyasapogenol B as an aglycone using the abovementioned protein

[0074] Examples of the "glycoside having soyasapogenol B as an aglycone" include soyasaponins I, II, III, IV, and V, azukisaponins II and V, astragaloside VIII, and sophoraflavoside I, which are primarily found in leguminous plants.

[0075] Examples of the substance containing a glycoside having soyasapogenol B as an aglycone include a substance extracted from soybeans or defatted soybeans (soybean cake) with hot water, alcohol or alcohol hydrate, or preferably a substance from which impurities such as proteins, sugars and lipids are removed by an ordinary method.

[0076] According to the present invention, soyasapogenol B can be obtained by allowing a culture containing a protein having saponin-decomposing activity, a protein according to the present invention or a protein obtained from a host according to the present invention to act on a substance containing a glycoside having soyasapogenol B as an aglycone and/or said glycoside.

[0077] More specifically, for example, about 1% to 10% by weight saponin (Koshiro Seiyaku) is dissolved in water or a buffer solution, such as an acetate buffer or a phosphate buffer, to which saponin-decomposing enzyme is added. The reaction is carried out at an appropriate temperature, for example 20°C to 50°C, after which the resulting reaction solution is extracted with an organic solvent such as ethyl acetate to obtain soyasapogenol B.

[EXAMPLES]

[0078] The present invention is further illustrated by the following examples that are not intended as a limitation of the invention.

Reference Example 1: Confirmation of Aspergillus saponin-decomposing enzyme

[0079] A PDA slant (about 1 cm^2) of Aspergillus sp. PF1224 (PERM BP-8004) was inoculated into 100 ml of a TS medium (2.0% soluble starch, 1.0% glucose, 0.5% polypeptone, 0.6% wheat germ, 0.3% yeast extract, 0.2% soybean grounds, and 0.2% calcium carbonate (pH 7.0 before sterilization)) dispensed into a 500-ml Erlenmeyer flask. Incubation was then carried out at 25°C for 3 days with shaking. The resulting culture (4 ml) was inoculated into 100 ml of an MY medium (4% malt extract, 2.0% yeast extract, 0.2% potassium dihydrogenphosphate, 0.2% ammonium sulfate, 0.03% magnesium sulfate heptahydrate, 0.03% calcium chloride dihydrate (pH 7.0)) supplemented with 4.0% soybean saponin (Koshiro Seiyaku) dispensed into a 500-ml Erlenmeyer flask and incubation was then carried out for 3 days with shaking.

[0080] Saponin-decomposing activity shown in Test Example 1 was used as an index in the purification of saponin-decomposing enzyme derived from Aspergillus hereinafter.

[0081] The resulting culture (about 800 ml) was filtered with a glass filter (G3) and then centrifuged (8,000 rpm, 30 minutes) to remove cell debris. Ammonium sulfate (294 g) was added to about 570 ml of the supernatant thus obtained and the resulting precipitate was recovered by centrifugation (8,000 rpm, 30 minutes). This precipitate was dissolved in about 120 ml of a buffer solution A (0.1 M sodium acetate buffer, 1 M ammonium sulfate (pH 5.8)) and the resulting solution was subjected to hydrophobic chromatography using Butyl Toyopearl 650S (26 mm i.d. x 330 mm) (Tosoh Co.). Elution was carried out with a concentration gradient from a buffer solution B (0.1 M sodium phosphate buffer-1 M ammonium sulfate (pH 5.8)) to 0.1 M sodium phosphate buffer (pH 5.8) and an unadsorbed fraction and a fraction eluted with an ammonium sulfate at a concentration from 1 M to 0.5 M were recovered.

[0082] Each of the recovered fractions was concentrated using Pellicon XL (cut-off molecular weight: 10,000) (Millipore), after which 1 M Tris-HCl buffer and ammonium sulfate were added to the concentrate so as to make their concentration the same as in a buffer solution C (50 mM Tris-HCl buffer, 1 M ammonium sulfate (pH 7.5)) and the resulting

solution was subjected to hydrophobic chromatography using 6 ml of Resource PHE (Amersham Biosciences). Elution was carried out with a concentration gradient from the buffer solution C to the 50 mM Tris-HCl buffer solution (pH 7.5) and an unadsorbed fraction was recovered.

[0083] The fraction thus obtained was concentrated using Ultrafree 15 (cut-off molecular weight: 5,000) (Millipore) and then subjected to gel filtration chromatography using Superdex 200 pg (16 mm i.d. × 600 mm) (Amersham Biosciences). Elution was carried out with a buffer solution D (25 mM sodium phosphate buffer, 0.15 M sodium chloride (pH 5.8)) and a fraction of a cut-off molecular weight of about 90 kDa was recovered.

[0084] SDS-PAGE was carried out with this fraction and a single band with an estimated molecular weight of about 90 kDa was observed.

Test Example 1: Measurement of saponin-decomposing activity

[0085] An enzyme solution containing an enzyme of interest was desalted using a PD-10 column (Amersham Biosciences), after which an equal volume of 2% saponin solution was mixed and reaction was carried out at 37°C for about 16 hours. The resulting reaction solution was extracted with an equal volume of ethyl acetate and the resulting extract was developed using TLC (solvent system used: chloroform:methanol = 95:5). Utilizing color reaction of vanillin-sulfuric acid, soyasapogenol B having an Rf value of 0.35 was detected to measure enzyme activity of the enzyme solution of interest.

Test Example 2: Quantitative analysis of saponin-decomposing activity

[0086] A diluted enzyme solution was added to 50 μl of a 2% saponin solution to make a total volume of 100 μl and the resulting admixture was reacted for 30 minutes. Next, the resulting reaction solution was extracted with an equal volume of ethyl acetate and a 50 μl portion of the extract was diluted with 450 μl of mobile phase. A 10 μl portion of the dilution was subjected to high performance liquid chromatography under the following conditions and a peak height at a retention time of about 7.5 minutes was measured. By comparing this height with that of authentic soyasapogenol B, saponin-decomposing activity of this enzyme was quantitatively evaluated.

Column : Inertsil ODS -2,5 μm (4.6 mm i.d × 250 mm)
Column temperature: 40°C
Mobile phase: acetonitrile:methanol:water = 50:35:15
Mobile phase flow rate: 0.8 ml/min

Example 1: Isolation and purification of saponin-decomposing enzyme derived from genus Neocosmospora (SDN)

[0087] A PDA slant (about 1 cm^2) of Neocosmospora vasinfecta var. vasinfecta PF1225 (FERM BP-7475) was inoculated into 100 ml of a TS medium dispensed into a 500-ml Erlenmeyer flask. Incubation was carried out at 25°C for 3 days with shaking. The resulting culture (4 ml) was inoculated into 100 ml of an MY medium supplemented with 4.0% soybean saponin (Koshiro Seiyaku) dispensed into a 500-ml Erlenmeyer flask and then incubation was carried out for 3 days with shaking.

[0088] Saponin-decomposing activity shown in Test Example 1 was used as an index in the purification of saponin-decomposing enzyme derived from genus Neocosmospora hereinafter.

[0089] The resulting culture (about 800 ml) was diluted with about 2 times volume of water and then centrifuged (8,000 rpm, 30 minutes) to remove cells. Ammonium sulfate (171 g) was added to the supernatant and the resulting precipitate was removed by centrifugation (8,000 rpm, 30 minutes). Further ammonium sulfate (573 g) was added to the resulting supernatant and the resulting precipitate was recovered by centrifugation (8,000 rpm, 30 minutes) and dissolved in 70 ml of a buffer solution C. The resulting solution was subjected to hydrophobic chromatography using Butyl Toyopearl 650S (26 mm i.d. × 110 mm) (Tosoh Co.). Elution was carried out with a concentration gradient from a buffer solution C to 50 mM Tris-HCl buffer (pH 7.5) and an unadsorbed fraction was recovered.

[0090] Ammonium sulfate (about 239 g) was added to about 500 ml of this fraction and the resulting precipitate was recovered by centrifugation. The recovered precipitate was then dissolved in 4 ml of a buffer solution B and the resulting solution was subjected to hydrophobic chromatography using Phenyl Sepharose FF (16 mm i.d. × 100 mm) (Amersham Biosciences). Elution was carried out with a concentration gradient from a buffer solution B to a 0.1 M sodium phosphate buffer solution (pH 5.8) and a fraction at an ammonium sulfate concentration of about 0.4 M was recovered.

[0091] The fraction thus recovered was subjected to gel filtration chromatography using Superdex 200 pg (16 mm i.d. × 600 mm) (Amersham Biosciences). Elution was carried out with a buffer solution E (50 mM Tris-HCl buffer, 0.15 M sodium chloride (pH 7.5)) and a fraction of a cut-off molecular weight of about 76,000 was recovered.

[0092] SDS-PAGE was carried out with this fraction and a single band of an estimated molecular weight of about 77 kDa was observed.

Example 2: Amino acid sequence analysis of saponin-decomposing enzyme (SDN)

2a) Amino acid sequence of the N-terminal side

[0093] The fraction prepared as in Example 1 was subjected to SDS-PAGE and blotted onto a PVDF membrane (Immobilon-PSQ) (Millipore), after which the membrane was washed and dried in air. This was subjected to a protein sequencer model 492 (Applied Biosystems) to analyze the amino acid sequence.

[0094] The amino acid sequence obtained by the analysis was as follows:
N-terminal amino acid sequence: ASPPASVPNNPSSEEITLQ (SEQ ID NO: 7)

2b) Analysis of inner amino acid sequence (peptide mapping)

[0095] The fraction prepared in Example 1 was subjected to SDS-PAGE and the resultant proteins were stained using Coomassie Brilliant Blue R250. A single band stained at an estimated molecular weight of about 77 kDa was excised and destained using a 0.2 M ammonium bicarbonate buffer solution (pH 8.0) in 50% acetonitrile and dried at room temperature for about 2 hours in air.

[0096] Next, this gel strip was immersed in a 0.2 M ammonium bicarbonate buffer solution (pH 8.0) containing 0.02% Tween 20, after which trypsin (Promega) was added and reaction was carried out at 37°C for 2 days. After the reaction, the supernatant was recovered and the gel strip was further washed 3 times with 60% acetonitrile and 0.1% trifluoracetic acid. The resulting washings and the reaction supernatant were combined, concentrated and subjected to a Model 172μ preparative HPLC system (Applied Biosystems) (RP-300 Aquiapore C18, 220 × 2.1 mm, with a concentration gradient from 0.1% trifluoracetic acid-35% acetonitrile to 0.085% trifluoracetic acid-35% acetonitrile). The following 5 polypeptides were fractionated.
Trp26.8: LVFNPSPK (SEQ ID NO: 8)
Trp27.59: WNVAADGSGPSGEIR (SEQ ID NO: 9)
Trp32.07: VTILHNPEGVAPITAK (SEQ ID NO: 10)
Trp39.43: EHSDTIPWGVPYVPGSQ (SEQ ID NO: 11)
Trp41.3: LTDYSFDWYSDIR (SEQ ID NO: 12)

Example 3: Cloning and sequence analysis of saponin-decomposing enzyme (SDN)

3a) Preparation of long probe using PCR

[0097] A genomic DNA was prepared from cultured cells of Neocosmospora vasinfecta var. vasinfecta PF1225 (FERM BP-7475) to be used as a template for PCR.

[0098] The genomic DNA was isolated according to the method of Horiuchi et al. (J. Bacteriol., 170, 272-278, 1988). First, cells cultured in a TS medium were recovered by centrifugation (7,500 rpm, 10 minutes). The cells thus obtained were lyophilized, suspended in a TE solution (10 mM Tris-HCl buffer, 1 mM EDTA (pH 8.0)) and then treated in a 3% SDS solution at 60°C for 30 minutes. Then TE-saturated phenol extraction was carried out to remove cell debris.

[0099] The extract was precipitated with ethanol and treated with Ribonuclease A (Nippon Gene) and Proteinase K (Wako Pure Chemical Industries, Ltd.), and the nucleic acid was then precipitated with 12% polyethylene glycol 6000. The precipitate was subjected to TE-saturated phenol extraction and ethanol precipitation, and the resulting precipitate was dissolved in a TE solution to obtain the genomic DNA.

[0100] Based on the peptide sequences obtained in Example 2, the following oligonucleotides encoding these sequences were synthesized and used as primers for PCR: Primer N1: CCIGCITCNGTNCCNAA (SEQ ID NO: 13)
Primer N2: CCIGCIAGYGTNCCNAA (SEQ ID NO: 14)
Primer 2A: CCRTCIGCNGCNACRTT (SEQ ID NO: 15)
Primer 3A: CCCCAIGGDATNGTRTC (SEQ ID NO: 16)
Primer 4A: ACICCYTCNGGRTTRTG (SEQ ID NO: 17)

[0101] The PCR was carried out using Takara Taq (Takara Shuzo Co., Ltd.). The fragments were amplified by repeating 10 cycles of 30 seconds at 94°C, 30 seconds at 45°C, and 3 minutes at 55°C, after heat denaturation at 94°C for 1 minute, followed by 20 cycles of 30 seconds at 94°C, 30 seconds at 47°C, and 3 minutes at 60°C. As a result, a specific fragment of about 0.8 kb was amplified in a combination of primer N1 and primer 4A. This fragment was cloned into pCR2.1-TOPO (pCR2.1-2) using a TOPO TA cloning kit (Invitrogen).

[0102] DNA sequence analysis was carried out using dRhodamine Terminator cycle sequencing ready reaction (Applied Biosystems) and ABI PRISM 310 genetic analyzer (Applied Biosystems). Decoding of the PCR product cloned in pCR2.1-2 revealed that this fragment was the amplification of the region from position 88 to position 812 of the sequence of SEQ ID NO: 1.

3b) Southern analysis and sequence decoding using inverse PCR

[0103] In Southern analysis, a genomic DNA digested with EcoRI was subjected to agarose gel electrophoresis and then to blotting onto Hibond N+ (Amersham Biosciences). An ECF Random-Prime Labelling and Detection System (Amersham Biosciences) was used for hybridization and a Molecular Imager FX (Bio-Rad) was used for band detection.

[0104] A band of about 2 kb was detected when the PCR product obtained in 3a) in Example 3 was used as a probe.

[0105] Next, the genomic DNA was digested with EcoRI, and a fragment of about 2 kb was recovered and circularized using a DNA ligation kit ver. 2 (Takara Shuzo Co., Ltd.). Using the resulting loop as a template, the fragment was amplified using LA Taq (Takara Shuzo Co., Ltd.) with the following primers for inverse PCR by repeating 25 cycles of a serial step consisting of 30 seconds at 94°C, 30 seconds at 50°C, and 4.5 minutes at 72°C, after heat denaturation at 94°C for 1 minute. This amplified fragment of about 2 kb was cloned using a TOPO TA cloning kit (Invitrogen) and its sequence was analyzed using primer walking.

Primer 1 for inverse PCR: TGACGCTGATACCAACGGCG (SEQ ID NO: 18)

Primer 2 for inverse PCR: CTAGTGGCAGTATTGGACAG (SEQ ID NO: 19)

3c) Determination of translation region using 3' RACE and 5' RACE methods

[0106] The translation region was determined by the 3' RACE and 5' RACE methods using cDNA as a template. cDNA was prepared as follows.

[0107] As described in Example 1, a one ml portion of the culture of Neocosmospora vasinfecta var. vasinfecta PF1225 (FERM BD-7475) cultured in a TS medium was inoculated into 100 ml of an MY medium supplemented with 1% soybean saponin dispensed into a 500-ml Erlenmeyer flask. After incubation at 25°C for 32 hours with shaking, cells were filtered through a nylon mesh (50 μm) and the cells thus obtained were frozen with liquid nitrogen. The frozen cells were smashed with a mortar and pestle and the whole RNA was extracted from the smashed cells. The whole RNA was extracted using ISOGEN (Nippon Gene) according to the attached protocol. mRNA was purified from the whole RNA using Oligotex-dT30

<Super> (Roche Diagnostics) according to the attached protocol.

[0108] By applying a 5'/3' RACE kit (Roche Diagnostics) to this mRNA, 3' and 5' regions were amplified using 3' RACE and 5' RACE according to the attached protocol. In this case, in the 3' RACE method, AmpriTaq Gold (Applied Biosystems) was used in the primary PCR and PCR Supermix High Fidelity (Lifetech Oriental Co., Ltd.) was used in the secondary PCR . In the primary and secondary PCRs in the 5' RACE method, PCR Supermix High Fidelity (Lifetech Oriental Co., Ltd.) was used.

[0109] The sequences of 3' RACE- and 5' RACE-specific primers were as follows.

3' RACE specific primer for primary PCR:
CCCAGGCCTTTAAGGATGGC (SEQ ID NO: 20)
3' RACE specific primer for secondary PCR:
CTAGTGGCAGTATTGGACAG (SEQ ID NO: 19)
5' RACE specific primer for cDNA synthesis:
TGACGCTGATACCAACGGCG (SEQ ID NO: 18)
5' RACE specific primer for primary PCR:
CTGCTTGAGGGTAATGGGCTC (SEQ ID NO: 21)
5' RACE specific primer for secondary PCR:
ACAGACGCCGGAGGPrGAAGCG (SEQ ID NO: 22)

[0110] The translation region of the SDN gene shown in SEQ ID NO: 1 was thus determined. From the result in Example 2a), the N terminal of the mature protein of SDN amino acid sequence was found to be located at position 27 from Met of the translation initiation site.

[0111] The presence of introns in the translation region was confirmed by comparing DNA sequences of the genomic DNA and cDNA. It was revealed that no intron was present in this translation region.

Example 4: Expression of saponin-decomposing enzyme (SDN) in Escherichia coli

[0112] PCR was carried out with primers for expression in E. coli shown below using the cDNA obtained in Example 3 as a template.

[0113] The translation region of the SDN gene was amplified using PCR Supermix High Fidelity (Lifetech Oriental Co., Ltd.) by repeating 25 cycles of a serial step consisting of 30 seconds at 94C, 30 seconds at 50C, and 2 minutes at 72C, after denaturation at 94°C for 1 minute. A fragment obtained by digesting the resulting product with restriction enzymes NdeI and BamHI was ligated to plasmid pET15b (Novagen, Inc.) digested with the same restriction enzymes, using a

DNA ligation kit ver. 2 (Takara Shuzo Co., Ltd.). E. coli strain BL21 (DE3) was transformed using this product according to an ordinary method and colonies having ampicillin resistance were obtained.
N-terminal primer for E. coli expression:
GGGCATATGGCTTCTCCTCCTGCTTCTG (SEQ ID NO: 23)
C-terminal primer for E. coli expression:
GGGGGATCCTTAAGTGCCGCTCTGAGGACTACG (SEQ ID NO: 24)

[0114] Colonies obtained were used for the following experiment.

[0115] Cells taken from the colonies were inoculated into 50 ml of an LB medium containing 50 μg/ml ampicillin dispensed in a 250-ml Erlenmeyer flask and incubation was carried out at 37°C overnight with shaking. A 2 ml portion of this culture was further inoculated into 50 ml of an LB medium containing 50 μg/ml ampicillin dispensed in a 250-ml Erlenmeyer flask and incubation was carried out at 37°C for 3 hours with shaking. Isopropyl-β-D-thiogalactopyranoside was added to the resulting culture at a final concentration of 0.4 mM and incubation was carried out for 3 hours for induction.

[0116] Cells thus cultured were collected by centrifugation and suspended in a buffer solution F (50 mM Tris-HCl buffer, 2 mM EDTA (pH 8.0)), after which cells were again recovered by centrifugation. After freezing at -80°C, these cells were suspended in 5 ml of a buffer solution F, lysozyme and Triton X 100 were added at final concentrations of 100 μg/ml and 0.1%, respectively, and the resulting suspension was allowed to stand at room temperature for about 20 minutes. Under ice cold, the cells were disrupted by ultrasonic treatment twice at a 50% duty cycle for 30 seconds using Sonifier 450 (BRANSON) and cell debris were removed by centrifugation.

[0117] Saponin-decomposing activity was measured according to Test Example 1 for the cell extract thus obtained as a crude enzyme solution.

[0118] As a result, a spot of the decomposed product, soyasapogenol B, was observed on TLC only for the extract of the cells in which the translation region of the saponin-decomposing enzyme was cloned.

Example 5: Expression of saponin-decomposing enzyme (SDN) in Trichoderma viride

5a) Construction of vector for transformation

[0119] PCR was carried out as described in Example 4 with primers for expression in Trichoderma shown below using the cDNA obtained in Example 3 as a template.

[0120] A fragment obtained by digesting the resulting PCR product with restriction enzymes SmaI and PstI was ligated to plasmid pCB1-M2 (see Example 5 of WO 98/11239) digested with StuI and PstI, using a DNA ligation kit ver. 2 (Takara Shuzo Co., Ltd). The product was digested with XbaI, dephosphorylated and then linked to an XbaI cassette of the pyr4 gene derived from Neurospora crassa to construct pCB-SBe (Figure 1).
N-terminal primer for expression in Trichoderma:
GGGCCCGGGGCGCATCATGCACTTCTTTGACAAAGCGAC (SEQ ID NO: 25)
C-terminal primer for expression in Trichoderma:
GGGCTGCAGTTAAGTGCCGCTCTGAGGACT (SEQ ID NO: 26)

[0121] The XbaI cassette of the pyr4 gene was constructed as follows.

[0122] pFB6 (Biochem. Biophys. Res. Commun., 112, 284-289, 1983) was first digested with BglII and then partially digested with HindIII to recover a fragment of about 1.9 kb. This fragment was ligated to pLITMUS28 (New England Biolabs) digested with BglII and HindIII: Next, the product was digested with BglII, blunted using a DNA blunting kit (Takara Shuzo Co., Ltd.), and then linked to a phosphorylated linker pXbaI (Takara Shuzo Co., Ltd.) to construct the XbaI cassette of the pyr4 gene.

5b) Acquisition of uracyl-requiring strain derived from Trichoderma viride

[0123] A spore suspension of Trichoderma viride MC300-1 (about 1.0×10^9 CFU/ml) was exposed to 2 UV lights at a distance of 30 cm with gentle mixing. The suspension was spread on a selective medium and incubated at 28°C for 7 days and then grown strains were selected.

[0124] A selective medium used was a minimum medium (0.2% potassium dihydrogenphosphate, 0.4% ammonium sulfate, 0.03% urea, 0.03% magnesium sulfate heptahydrate, 0.03% calcium chloride, 0.5% glucose, 2.5% agar, 0.01% trace elements (5 mg of ion sulfate heptahydrate, 1.56 mg of manganese sulfate heptahydrate, 1.4 mg of zinc sulfate heptahydrate, and 2.0 mg of cobalt chloride dissolved in 1 L of water) supplemented with 10 ug/ml uridine and 1.0 mg/ml 5-fluoroorotic acid.

5c) Transformation of Trichoderma viride and detection of saponin-decomposition activity of each recombinant

[0125] Cells of the uracyl-requiring Trichoderma viride strain obtained in 5b) of Example 5 were inoculated into 50 ml

of a cell forming medium(1.0% yeast extract, 1.0% molt extract, 2.0% polypeptone, 2.5% glucose, 0.1% potassium dihydrogenphosphate, 0,05% magnesium sulfate heptahydrate, (pH 7.0 before sterilization)) dispensed in a 200-ml Erlenmeyer flask and incubation was carried out at 28°C for 2 days with shaking. Mycelia were recovered from the resultant culture by centrifugation. Next, protoplasts were prepared from the mycelia, after which a DNA solution of plasmid pCB-SBe was added to carry out transformation (see Example 7 of WO 98/11239).

[0126] Further, in regeneration of transformants, 0.5 M sucrose was added to the minimum medium. Grown colonies were again inoculated onto the minimum medium and colonies grown in the medium were recognized as transformants.

[0127] Plasmid pCB-SBe was introduced into the uracyl-requiring Trichoderma viride strain. As a result, 3 transformants per 1 μg of pCB-SBe were obtained. Each of the transformants, 25 strains, was cultured (see Example 1 of WO 98/11239) and the resultant culture supernatant was subjected to SDS-PAGE, on which a band showing a molecular weight of about 68 kDa was observed only for the transformants.

[0128] Saponin-decomposing activity was measured according to Test Example 1 for this culture supernatant. As a result, a spot of the decomposed product, soyasapogenol B, was observed on TLC. On the other hand, this spot was not observed for the parent strain, the uracyl-requiring Trichoderma viride.

5d) Purification of recombinant saponin-decomposing enzyme (recombinant SDN), and comparison of its activity with wild-type saponin-decomposing enzyme derived from Neocosmopora vasinfecta var. vasinfects PF1225 (wild-type SDN)

[0129] The culture obtained in 5c) of Example 5 (about 700 ml) was centrifuged (8,000 rpm, 30 minutes) to remove cell debris. Ammonium sulfate (64 g) was added to about 560 ml of the supernatant thus obtained and the resulting precipitate was removed by centrifugation (8,000 rpm, 30 minutes). Further, 74 g of ammonium sulfate were added to about 600 ml of the resultant supernatant and the resultant precipitate was recovered. To this precipitate were added 100 ml of 0.05 M Tris-HCl buffer (pH 7.5) and 16 g of ammonium sulfate and the admixture was subjected to hydrophobic chromatography using Butyl Toyopearl 650S (26 mm i.d. x 330 mm) (Tosoh Co.). Elution was carried out with a concentration gradient from a buffer solution C to 50 mM Tris-HCl buffer (pH 7.5) and an unadsorbed fraction and a fraction eluted at an ammonium sulfate concentration from 1 M to 0.6 M were recovered.

[0130] Each of the fraction thus obtained was concentrated using Pellicon XL (cut-off molecular weight: 10,000) (Millipore). To about 8 ml of this concentrate were added 1.3 g of ammonium sulfate and 2 ml of 0.5 M sodium phosphate buffer (pH 5.8) and the admixture was subjected to hydrophobic chromatography using 6 ml of Resource PHE (Amersham Biosciences). Elution was carried out with a concentration gradient from a buffer solution B to 0.1 M sodium phosphate buffer (pH 5.8) and an unadsorbed fraction was recovered.

[0131] The fraction thus obtained was concentrated using Ultrafree 15 (cut-off molecular weight: 5,000) (Millipore). This concentrate was subjected to gel filtration chromatography using Superdex 200 pg (16 mm i.d. × 600 mm) (Amersham Biosciences). Elution was carried out with a buffer solution G (50 mM sodium phosphate buffer, 0.15 M sodium chloride (pH 7.0)) and a fraction of a cut-off molecular weight of about 68,000 was recovered.

[0132] SDS-PAGE was carried out with this fraction and a single band with an estimated molecular weight of about 68 kDa was observed.

[0133] The optimum pH and the optimum temperature for the recombinant saponin-decomposing enzyme thus purified (occasionally referred to as "recombinant SDN" hereinafter) and the saponin-decomposing enzyme purified in Example 1 (occasionally referred to as "wild-type SDN" hereinafter) were measured according to Test Example 2.

[0134] In measuring the optimum pH, first, to 50 μl of a 2% saponin solution were added 20 μl each of 0.5 M individual buffer solutions (sodium acetate buffer (pH 4.5, pH 5.0, pH 5.8); sodium phosphate buffer (pH 5.0, pH 5.8, pH 7.0) and Tris-HCl buffer (pH 7.0, pH 8.0, pH 9.0)) and a diluted enzyme solution to make a total volume of 100 μl. Reaction was carried out at 37°C for 30 minutes, and then the amount of soyasapogenol B produced was measured.

[0135] Results are shown in Figure 2.

[0136] In measuring the optimum temperature, first, to 50 μl of a 2% saponin solution were added 20 μl of 0.5 M sodium phosphate buffer (pH 5.8) and a diluted enzyme solution to make a total volume of 100 μl. Reaction was carried out at each specified temperature for 30 minutes, and then the amount of soyasapogenol B produced was measured.

[0137] Results are shown in Figure 3.

[0138] As evident from these results, there was not much difference in activity although there was some difference in the molecular weight determined by SDS-PAGE.

Example 6: Amino acid sequence analysis for saponin-decomposing enzyme derived from Aspergillus sp. PF1224 (SDA)

[0139] saponin-decomposing enzyme purified from Aspergillus sp. PF1224 (FERM BP-8004) (SDA) (Reference Example 1) was fragmented as described in 2b) of Example 2 after excising a band of about 90 kDa, and subjected to HPLC as described in 2b) in Example 2 to fractionate the following 4 kinds of peptides.

Trp23.67: LYNPDSPQPISAK (SEQ ID NO: 27)
Trp24.0: LQFNPAPK (SEQ ID NO: 28)
Trp38.05: VDWFSDLTSTGQVTGSK (SEQ ID NO: 29)
Trp24.5: GEVSGSASVSIIHD (SEQ ID NO: 30)

Example 7: Cloning and sequence analysis of SDA gene

7a) Preparation of long probe using PCR

[0140] A genomic DNA was isolated from cells cultured as described in Reference Example 1, as described in Example 3.

[0141] Based on the peptide sequences obtained in Example 6, the following oligonucleotides encoding these sequences were synthesized and used as primers for PCR. Primer 23.67s1: TAYAAYCCIGAYTCNCC (SEQ ID NO: 31)
Primer 23.67s2: TAYAAYCCNGAYAGYCC (SEQ ID NO: 32)
Primer 24.0s : CARTTYAAYCCIGCNCC (SEQ ID NO: 33)
Primer 24.0a : GGIGCNGGRTTRAAYTG (SEQ ID NO: 34)
Primer 38.05a1 AARTCNGARAACCARTC (SEQ ID NO: 35)
Primer 38.05a2: AARTCRCTRAACCARTC (SEQ ID NO: 36)

[0142] The PCR was carried out as described in 3a) in Example 3.

[0143] As a result, a fragment of about 1 kb was specifically amplified in a combination of primer 24.0s and primer 38.05a1 among the primers above, which was then cloned into pCR2.1-TOPO using a TOPO TA cloning kit (Invitrogen) (pSDAPCR1)

[0144] Results of sequence analysis revealed that the fragment cloned into pSDAPCR1 was the amplification of the region from position 709 to position 1748 of the sequence of SEQ ID NO: 3.

7b) Southern analysis and screening using E. coli colony library

[0145] In Southern analysis, a genomic DNA previously digested with BamHI, EcoRI, and HindIII was subjected to agarose gel electrophoresis and then to blotting onto Hibond N+ (Amersham Bioscience). An ECF Random-Prime Labelling and Detection System (Amersham Bioscience) was used for hybridization and a Molecular Imager FX (Bio-Rad) was used for band detection.

[0146] Bands for a BamHI fragment of about 10 kb, an EcoRI fragment of about 20 kb, and a HindIII fragment of about 5 kb were detected when the PCR products obtained in 7a) above were used as a probe.

[0147] Next, the genomic DNA was digested with HindIII, and fragments of about 4 kb to 6 kb were recovered. The product was then linked to pUC18, which was previously digested with HindIII and dephosphorylated, to transform an E. coli strain DH5α. This E. coli was grown on an LB agar medium supplemented with ampicillin for colony formation, about 1,000 colonies thus obtained were blotted onto Hibond N+ (Amersham Bioscience). Here, one kind of positive clone (pSDAHind5/18) was obtained using the PCR product obtained in the abovementioned 7a) as a probe. This clone contained a HindIII fragment of about 5 kb.

7c) Determination of translation region using 3' RACE and 5' RACE methods

[0148] As described in 3c) in Example 3, the whole RNA was extracted from culture cells of Aspergillus sp. PF1224 (FERM BP-8004) prepared in Reference Example 1 and further, mRNA was isolated using a QuickPrep mRNA purification kit (Amersham Bioscience) according to the attached protocol.

[0149] By applying a 5'/3' RACE kit (Roche Diagnostics) to this mRNA, 3' and 5' regions were amplified. LA Taq (Takara Shuzo Co., Ltd.) was used for each PCR.

[0150] Sequences of 3'RACE-and 5' RACE-specific primers were as follows.
3' RACE specific primer for primary PCR:
CCTCGATACCCGAGGGACCG (SEQ ID NO: 37)
3' RACE specific primer for secondary PCR:
GATGGGTTGCATGTTATCGC (SEQ ID NO: 38)
5' RACE specific primer for cDNA synthesis:
GCGATAACATGCAACCCATC (SEQ ID NO: 39)
5' RACE specific primer for primary PCR:
GACCACCTGGTTCAGTGGTG (SEQ ID NO: 40)
5' RACE specific primer for secondary PCR:
GGGTTATAGAGTCTGGTAACG (SEQ ID NO: 41)

[0151] The translation region of the SDA gene shown in SEQ ID NO: 3 was thus determined. The SDA protein purified as Reference Example 1 was analyzed for the mature N-terminal amino acid sequence in the same manner as described in Example 2a). As a result, the N terminal of the mature protein of SDA amino acid sequence was found to be located at position 29 from Met of the translation initiation site. Further, the presence of introns in the translation region was confirmed by comparing DNA sequence of the genomic RNA and cDNA. It was revealed that no intron was present in this translation region.

Example 8: Expression of saponin-decomposing enzyme derived from Aspergillus sp. PF1224 (SDA) in Trichoderma viride

8a) Construction of vector for transformation

[0152] First, PCR was carried out as described in Example 4 with primers for expression in Trichoderma shown below using pSDAHind5/18 obtained in Example 7b as a template.

[0153] A fragment obtained by digesting the resulting PCR product with restriction enzymes StuI and XhoI was ligated to plasmid pCB1-M2 (see Example 5 of WO 98/11239) previously digested with StuI and XhoI using a DNA ligation kit ver. 2 (Takara Shuzo Co., Ltd.) to construct pCB-SDAe (Figure 4).
N-terminal primer for SDA expression in Trichoderma:
GGGAGGCCTGCGCATCATGCATGTTGTCGCAAGTACCAC (SEQ ID NO: 42)
C-terminal primer for SDA expression in Trichoderma:
GGGCTCGAGTACCTCAAGTCCCATTTGCCGGCTGC (SEQ ID NO: 43)

8b) Transformation of Trichoderma viride and detection of saponin-decomposing activity of each recombinant

[0154] A host, the uracyl-requiring Trichoderma viride strain obtained in 5b) in Example 5, was transformed by the co-transformation method using pCB-SDAe and vector pPYR4 in which the pyr4 cassette was ligated to pLITMUS28 (see 5a) in Example 5), as described in 5c) in Example 5. As a result, about 12 strains of transformants per 1 μg of DNA were obtained.

[0155] Each of the transformants thus obtained was cultured (see Example 1 of WO 98/11239) and the resultant culture supernatant was subjected to SDS-PAGE, on which a band showing a molecular weight of about 80 kDa was observed only for the transformants.

[0156] Saponin-decomposing activity was measured using this culture supernatant as described in Test Example 1. As a result, a spot of the decomposed product, soyasapogenol B, was observed on TLC.

8c) Purification of recombinant saponin-decomposing enzyme derived from Aspergillus sp. PF1224 (recombinant SDA), and comparison of its activity with wild-type saponin-decomposing enzyme derived from Aspergillus sp PF1224 (wild-type SDA)

[0157] The culture obtained in 8b) above (about 600 ml) was centrifuged (8,000 rpm, 30 minutes) to remove cell debris. Ammonium sulfate (57 g) was added to about 500 ml of the supernatant thus obtained and the resulting precipitate was removed by centrifugation. Further, 64 g (40% saturation fraction) and then 70 g (60% saturation fraction) of ammonium sulfate were added to this supernatant and the resultant precipitate was dissolved in a 0.1 M sodium phosphate buffer solution (pH 5.8). To the 60% saturation fraction was added ammonium sulfate to make a final concentration of 1M, and then the admixture was subjected to hydrophobic chromatography using Butyl Toyopearl 650S (26 mm i.d. × 250 mm) (Tosoh Co.). Elution was carried out with a concentration gradient from a buffer solution A to a 0.1 M sodium phosphate buffer solution (pH 5.8) and a fraction eluted at an ammonium sulfate concentration from 0.9 M to 0.2 M was recovered.

[0158] The fraction thus obtained was concentrated using Pellicon XL (cut-off molecular weight: 10,000) (Millipore) and Ultrafree 15 (cut-off molecular weight: 10,000) (Millipore), desalted using a PD-10 column (Amersham Bioscience) and then subjected to ion-exchange chromatography using 6 ml of Resource Q (Amersham Bioscience). Elution was carried out with a concentration gradient from 50 mM Tris-HCl buffer (pH 7.5) to 50 mM Tris-HCl buffer-0.5 M sodium chloride (pH 7.5), and an unadsorbed fraction and a fraction eluted at a salt concentration of 0.08 M were recovered.

[0159] The fraction thus obtained was concentrated using Ultrafree 15 (cut-off molecular weight: 10,000) (Millipore) and then subjected to gel filtration chromatography using Superdex 200 pg (16 mm i.d. x 600 mm) (Amersham Bioscience). Elution was carried out with a buffer solution G and a fraction of a cut-off molecular weight of about 50 kDa was recovered.

[0160] SDS-PAGE was carried out with this fraction and a single band with an estimated molecular weight of about 80 kDa was observed. Further, the cut-off molecular weight on the gel filtration and the molecular weight on the SDS-PAGE were different probably because this protein was adsorbed unspecifically to the carriers.

[0161] The optimum pH and the optimum temperature were measured for the recombinant SDA thus purified and the

saponin-decomposing enzyme purified in Reference Example 1 (wild-type SDA), according to Test Example 2.

[0162] The optimum pH and the optimum temperature were measured as described in 5d) in Example 5.

[0163] Results are shown in Figures 5 and 6.

[0164] As a result, it was revealed that although the recombinant SDA exhibited lower specific activity in sodium phosphate buffer at pH 7 as compared to the wild-type SDA, it exhibited improved specific activity in Tris-HCl buffer and at high pHs.

Example 9: Isolation and purification of saponin-decomposing enzyme derived from Eupenicillium brefeldianum PF1226 (SDE)

[0165] A PDA slant (about 1 cm^2) of Eupenicillium brefeldianum PF1226 (FERM BP-7476) was inoculated into 100 ml of a TS medium dispensed into a 500-ml Erlenmeyer flask and then incubation was carried out at 25°C for 3 days with shaking. The resulting culture (4 ml) was inoculated into 100 ml of an MY medium supplemented with 1.0% soybean saponin (Koshiro Seiyaku) dispensed into a 500-ml Erlenmeyer flask and then incubation was carried out for 7 days with shaking.

[0166] The resulting culture (about 1,000 ml) was filtered with a glass filter (G3) to remove cell debris. Ammonium sulfate (73 g) was added to this culture supernatant (about 640 ml) and the resulting precipitate was removed by centrifugation (8,000 rpm, 30 minutes). Further, ammonium sulfate (256 g) was added to the resulting supernatant (about 670 ml) and the resulting precipitate was recovered by centrifugation. This precipitate was dissolved in about 50 ml of 0.1 M sodium acetate buffer (pH 5.8), 13.2 g of ammonium sulfate was added, and then water was added to make a final concentration of 1 M ammonium sulfate-0.1M sodium acetate buffer.

[0167] After centrifugation, this solution was subjected to hydrophobic chromatography using Butyl Toyopearl 650S (26 mm i.d. × 330 mm) (Tosoh Co.). Elution was carried out with a concentration gradient from a buffer solution C to 50 mM Tris-HCl buffer and a fraction eluted at an ammonium sulfate concentration of 0.7 M to 0.5 M was recovered.

[0168] The recovered fraction was concentrated using Pellicon XL (cut-off molecular weight: 10,000) (Millipore), after which sodium phosphate buffer and ammonium sulfate were added to make the concentrate having the same component as the buffer solution A and the resulting solution was subjected to hydrophobic chromatography using 6 ml of Resource PHE (Amersham Bioscience). Elution was carried out with a concentration gradient from a buffer solution A to a 0.1 M sodium phosphate buffer solution (pH 5.8) and a fraction eluted from an ammonium sulfate concentration of 1 M to 0.3 M was recovered.

[0169] This fraction was concentrated using Ultrafree 15 (cut-off molecular weight: 10,000) (Millipore), desalted using a PD-10 column (Amersham Bioscience) and then subjected to ion-exchange chromatography using 6 ml of Resource Q (Amersham Bioscience). Elution was carried out with a concentration gradient from 20 mM sodium phosphate buffer (pH 7.0) to 20 mM sodium phosphate buffer-1 M sodium chloride (pH 7.0), and an unadsorbed fraction was recovered.

[0170] The fraction thus obtained was concentrated using Ultrafree 15 (cut-off molecular weight: 10,000) (Millipore) and then subjected to gel filtration chromatography using Superdex 200 pg (16 mm i.d. × 600 mm) (Amersham Bioscience). Elution was carried out with a buffer solution G and a fraction of a cut-off molecular weight of about 90 kDa was recovered.

[0171] SDS-PAGE was carried out with this fraction and a single band with an estimated molecular weight of about 90 kDa was observed.

Example 10 : Analysis of amino acid sequence of SDE

10a) Amino acid sequence of N-terminal side

[0172] The N-terminal side amino acid sequence of the fraction prepared in Example 9 was analyzed as described in 2a) in Example 2. As a result, the following amino acid sequence was obtained.
N-terminal amino acid sequence: STTPAPPQPEPI (SEQ ID NO: 44)

10b) Peptide mapping

[0173] The SDE purified in Example 9 was fragmented after excising a band of about 90 kDa, as described in 2b) in Example 2. The fragments were subjected to HPLC as described in 2b) in Example 2 and the following 3 kinds of peptides were fractionated.
Trp20.73: ADPAFSPDGTR (SEQ ID NO: 45)
Trp34.21: LHPDDTHMGWSSF (SEQ ID NO: 46)
Trp36.26: GFSGAGDEILYIGSTR (SEQ ID NO: 47)

Example 11: Cloning and sequence analysis of SDE gene

11a) Preparation of long probes using PCR

[0174] The genomic DNA was isolated from the cells cultured in Example 9, as described in Example 3.

[0175] Based on the sequences of the peptides obtained in Example 10, the following oligonucleotides encoding these sequences were synthesized and used as primers for PCR.

Primer Ns: CCICARCCNGARCCNAT (SEQ ID NO: 48)

Primer 20.37a: CTRAAIGCNGGRTCNGC (SEQ ID NO: 49)

Primer 34.21a: CCANCCCATRTGNGTRTC (SEQ ID NO: 50)

[0176] The PCR was carried out as described in 3a) in Example 3.

[0177] As a result, a fragment of about 1 kb was specifically amplified with a combination of primer Ns and primer 20.73a among the primers above. This fragment was cloned into pCR2.1-TOPO using a TOPO TA cloning kit (Invitrogen) (pSDEPCR5).

[0178] Results of sequence analysis revealed that the fragment cloned into pSDEPCR5 was the amplification of the region from position 70 to position 1247 of the sequence of SEQ ID NO: 5.

11b) Screening using Southern analysis and phage library

[0179] In Southern analysis, a genomic DNA digested with PstI, SphI and XhoI was subjected to agarose gel electrophoresis and then to blotting onto Hibond N+ (Amersham Bioscience). An ECF Random-Prime Labelling and Detection System (Amersham Bioscience) was used for hybridization and a Molecular Imager FX (Bio-Rad) was used for band detection.

[0180] Bands of a PstI fragment of about 3 kb, an SphI fragment of about 4 kb, and an XhoI fragment of about 6 kb were detected when the PCR product obtained in 11a) above was used as a probe.

[0181] Next, the genomic DNA was partially digested with Sau3A1. The resultant product was then linked to λEMBL3/BamHI vector (Stratagene) and packaged using a MaxPlax packaging extract kit (Epicentre Technologies). The resultant phage library (about 5×10^4 PFU) was blotted onto Hibond N+ (Amersham Bioscience) and then 5 kinds of positive clones were obtained using the PCR fragment cloned in pSDEPCR5 as a probe, according to a DIG Hi-Prime DNA Labelling and Detection Kit (Roche Diagnostics). Of these clones, a XhoI fragment was recovered from a phage DNA containing the 6 kb XhoI fragment and cloned into pBluescript II KS+ (pSDEXho/IIKS+1).

[0182] The DNA sequence of the SDE translation region was determined as shown in SEQ ID NO: 5 by the transposon method using pSDEXho/IIKS+1 as a template.

[0183] As a result of Example 10a), the N terminal of the mature protein of SDE amino acid sequence was found to be located at position 18 from Met of the translation initiation site.

[0184] The presence of introns in the translation region was confirmed by comparing the sequences of the genomic DNA and cDNA. It was revealed that no intron was present in the translation region.

Example 12: Expression of saponin-decomposing enzyme derived from Eupenicillium brefeldianum PF1226 (SDE) in Trichoderma viride

12a) Construction of vector for transformation

[0185] PCR was carried out with primers for Trichoderma secretion shown below using pSDEXho/IIKS+1 obtained in Example 11 as a template, as described in Example 4.

[0186] A fragment obtained by digesting the resultant PCR product with restriction enzymes SmaI and XhoI was ligated to plasmid pCB1-M2 (see Example 5 of WO 98/11239) previously digested with SmaI and XhoI, using a DNA ligation kit ver. 2 (Takara Shuzo Co., Ltd) to construct pCB-SDEs (Figure 7).

N-terminal primer for SDE secretion in Trichoderma:

GGGCCCGGGCTCAGACTACCCCGGCACCTCCTCAGCC (SEQ ID NO: 51)

C-terminal primer for SDE secretion in Trichoderma:

GGGCTCGAGTACCTCATGCACCATTGAGCGGCTGGTGG (SEQ ID NO: 52)

12b) Transformation of Trichoderma viride and detection of saponin-decomposing activity of each recombinant

[0187] Host cells of the uracyl-requiring Trichoderma viride strain obtained in 5b) of Example 5 were transformed by the co-transformation method using pCB-SDEs and vector pPYR4 in which the pyr4 cassette was linked to pLITMUS28 (see 5a) above), as described in 5c) in Example 5. As a result, about 28 strains of transformants per 1μg of DNA were

obtained.

[0188] Each of the transformants thus obtained was cultured (see Example 1 of WO 98/11239) and the resultant culture supernatant was subjected to SDS-PAGE, on which a band with an estimated molecular weight of about 67 kDa was observed only for the transformants.

[0189] Saponin-decomposing activity was measured as described in Test Example 1 for this culture supernatant. As a result, a spot of the decomposed product, soyasapogenol B, was observed on TLC.

12c) Purification of recombinant saponin-decomposing enzyme derived from Eupenicillium brefeldianum PF 1226 (recombinant SDE), and comparison of its activity with the wild-type saponin-decomposing enzyme derived from Eupenicillium brefeldianum PF1226 (wild-type SDE)

[0190] The culture obtained in 12b) in Example 12 (about 900 ml) was centrifuged (8,000 rpm, 30 minutes) to remove cell debris. Ammonium sulfate (78.7 g) was added to about 690 ml of the supernatant thus obtained and the resulting precipitate was removed by centrifugation. Further, 88.6 g of ammonium sulfate (40% saturation fraction) were added to the resultant supernatant and the resultant precipitate was dissolved to make 120 ml of 1 M ammonium sulfate-0.1 M sodium phosphate buffer (pH 5.8). A 20 ml portion of this solution was subjected to hydrophobic chromatography using Butyl Toyopearl 650S (26 mm i.d. × 330 mm) (Tosoh Co.). Elution was carried out with a concentration gradient from a buffer solution A to a 0.1 M sodium phosphate buffer solution and a fraction eluted at an ammonium sulfate concentration from 0.2 M to 0 M was recovered.

[0191] The fraction thus obtained was concentrated using Pellicon XL (cut-off molecular weight: 10,000) (Millipore) and Ultrafree 15 (cut-off molecular weight: 5,000) (Millipore), desalted using a PD-10 column (Amersham Bioscience) and then subjected to ion-exchange chromatography using 6 ml of Resource Q (Amersham Bioscience). Elution was carried out with a concentration gradient from 50 mM Tris-HCl buffer (pH 7.5) to 50 mM Tris-HCl buffer-0.5 M sodium chloride (pH 7.5), and a fraction eluted at a salt concentration from 0 M to 0.1 M was recovered.

[0192] The fraction thus obtained was concentrated using Ultrafree 15 (cut-off molecular weight: 5,000) (Millipore) and then subjected to gel filtration chromatography using Superdex 200 pg (16 mm i.d. x 600 mm) (Amersham Bioscience). Elution was carried out with a buffer solution G and a fraction of a cut-off molecular weight of about 50 kDa was recovered.

[0193] SDS-PAGE was carried out with this fraction and a single band with an estimated molecular weight of about 67 kDa was observed. Further, the cut-off molecular weight on the gel filtration and the molecular weight on the SDS-PAGE were different probably because this protein was adsorbed unspecifically to the carriers.

[0194] The optimum pH and the optimum temperature for the recombinant SDE thus purified and the saponin-decomposing enzyme purified in Example 9 (wild-type SDE) were measured according to Test Example 2.

[0195] The optimum pH and the optimum temperature were measured as described in 5d) in Example 5.

[0196] Results are shown in Figures 8 and 9.

[0197] As a result, it was revealed that the recombinant SDE exhibited improved activity in a Tris-HCl buffer solution and also at high pHs as compared to the wild-type SDE.

SEQUENCE LISTING

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-10                 -5                  -1  1                   5

Val Pro Asn Pro Pro Ser Pro Glu Pro Ile Thr Leu Lys Gln Leu Pro
                10                  15                  20

Leu Pro Pro Ile Ser Pro Ser Asp Asp Val Gly Ala Cys Thr Lys Gln
            25                  30                  35

Ile Asn Ser Arg Gly Thr Gly Cys Leu Ala Asn Gly Val Phe Glu Thr
        40                  45                  50

Phe Gln Ser Gly Asp Phe Leu Pro Asp Gly Lys His Val Ile Ala Met
    55                  60                  65                  70

Val Asn Phe Thr Gly Ala Pro Ala Ala Pro Ala Ala Gly Ser Ile Tyr
                75                  80                  85
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Ser Gly Pro Gln Val Ile Ile Val Lys Thr Asp Gly Lys Thr Phe Pro
90 95 100

Asn Gly Asp Pro Trp Lys Cys Ile Thr Cys Gly Val Pro Glu Lys Asn
105 110 115

Ala Val Gly Ile Ser Val Lys Tyr Asp Tyr Pro Gln Ala Phe Lys Asp
120 125 130

Gly Lys Arg Leu Leu Ile Gly His Asn Ile Leu Asp Cys Gly Thr Asn
135 140 145 150

Gln Leu Thr Ser Glu Ser Cys Lys Pro Asp Asn Thr His Ile Tyr Pro
155 160 165

Ile Arg Trp Asn Val Ala Ala Asp Gly Ser Gly Pro Ser Gly Glu Ile
170 175 180

Arg Glu Leu Arg Leu His Pro Asp Asn Val His Leu Glu Phe Ser Ser
185 190 195

Phe Thr Phe Ala Ser Gly Ser Ile Gly Gln Tyr Ala Tyr Phe Ser Arg
200 205 210

Leu Val Phe Asn Pro Ser Pro Lys Thr Gly Thr Pro Leu Ala Pro Arg
215 220 225 230

Tyr Asp Leu Glu Lys Val Thr Ile Leu His Asn Pro Glu Gly Val Ala
235 240 245

Pro Ile Thr Ala Lys Gly Lys Val Leu Ser Leu Asn Pro Gln Ala Ile
250 255 260

Ser Val Gly Glu Ala Arg Gly Phe Asn Gly Asp Gly Thr Glu Leu Thr
265 270 275

Tyr Val Gly Ser Asn Ile Glu Ser Cys Asn Asn Asp Val Phe Ala Val

280 285 290

His Leu Gln Thr Gly Val Val Arg Arg Leu Thr Asn His Pro Glu Tyr
295 300 305 310

Pro Asp Pro Leu Ala Phe Ser Pro Asp Asn Lys Trp Met Ala Val Met
315 320 325

Asp Thr Arg Gly Ser Gly Arg Asn Met Phe Ile Ala Gly Met Arg Gly
330 335 340

Ile Pro Pro Leu Val Asp Ile Val Gly Gly Ile Leu Pro Ala Ser Ser
345 350 355

Arg Asn Asn Gly Leu Arg Arg Phe Phe Gln Pro Tyr Leu Leu Asp Phe
360 365 370

Tyr Gly Asp Arg Gly Asp Tyr Tyr Gly Gln Lys Ile Asn Gly Asp Asn
375 380 385 390

Asn Gly Val Pro Gly Ser Gly Ala Ile Asn Asp Pro Glu Trp Asn Gly
395 400 405

Met Ala Asp Pro Arg Trp Ser Pro Asp Ser Arg Gln Leu Val Phe Trp
410 415 420

Gln Thr His Thr Val Ser Pro Ser Cys Gly Gly Ala Asn Pro Leu Pro
425 430 435

Cys Tyr Pro Ser Lys Glu Gln Gly Gly Arg Asn Tyr Arg Met Tyr Ile
440 445 450

Ala Thr Phe Thr Ser Arg Ser Pro Ser Pro Pro Ala Pro Val Lys Glu
455 460 465 470

His Ser Asp Thr Ile Pro Trp Gly Val Pro Tyr Val Pro Gly Ser Gln
475 480 485

Val Thr Pro Lys Pro Gly Leu Ala Gly Gly Ile Tyr Thr Leu Tyr Gly
490 495 500

Lys Ala Ser Gly Glu Ala Lys Val Asn Ile Thr Trp Gly Glu Ala Pro
505 510 515

Glu Ile Gly Thr Val Ser Val Val Tyr Lys Asp Tyr Ser Leu Asp Gly
520 525 530

Lys Ser Phe Leu Asn Gly Asn Glu Ser Val Thr Gly Ser Val Glu Arg
535 540 545 550

Leu Thr Asp Tyr Ser Phe Asp Trp Tyr Ser Asp Ile Arg Gln Thr Gly
555 560 565

Ala Val Lys Gly Thr Lys Lys Thr Ser Pro Gly Gly Phe His Ala Asn
570 575 580

Ile Asp Val Met Ile Asn Asp Leu Thr Ser Thr Gly Thr Leu Thr Thr
585 590 595

Thr Leu Asp Gly Val Glu Trp Arg Ser Pro Gln Ser Gly Thr
600 605 610

<210> 3
<211> 1902
<212> DNA
<213> Aspergillus sp. PF1224

<220>
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<222> (1).. (1899)

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<221> mat_peptide
<222> (85).. (1899)

<400> 3

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Met His Val Val Ala Ser Thr Thr Ala Phe Leu Gly Val Val Ser Thr
            -25                 -20                 -15

gtt gct ggg gta cac cat gtc aac aga gac acc agt caa cag atc cta   96
Val Ala Gly Val His His Val Asn Arg Asp Thr Ser Gln Gln Ile Leu
        -10                 -5              -1  1

aaa cca cct gta cca gag cct att gtc gtc acc gag ctt ccc ttg cct   144
Lys Pro Pro Val Pro Glu Pro Ile Val Val Thr Glu Leu Pro Leu Pro
  5                 10                  15                  20

cct gtc gcc gac agt aag gag ggc tct tgt act ccc gaa gtt agc cct   192
Pro Val Ala Asp Ser Lys Glu Gly Ser Cys Thr Pro Glu Val Ser Pro
                25                  30                  35

cac agg acc ggt tgt cta ctc aaa tcc tcc cag att cag agt gga aat   240
His Arg Thr Gly Cys Leu Leu Lys Ser Ser Gln Ile Gln Ser Gly Asn
            40                  45                  50

ttc ctt cct gac aac aat cat gtc ctt gtc agc tta aac ttc tca ggg   288
Phe Leu Pro Asp Asn Asn His Val Leu Val Ser Leu Asn Phe Ser Gly
        55                  60                  65

gct cca gca gct cca gat ccg gct agc att tat aat ggt acc cat ttg   336
Ala Pro Ala Ala Pro Asp Pro Ala Ser Ile Tyr Asn Gly Thr His Leu
    70                  75                  80

act ctg ata aag gct gat ggg acc aac ttt cct agt ggt gac cca tgg   384
Thr Leu Ile Lys Ala Asp Gly Thr Asn Phe Pro Ser Gly Asp Pro Trp
 85                  90                  95                  100

aag tgt att acc tgc ggc gtg ccg gaa gaa aac aag gtc ggc agc aca   432
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Lys Cys Ile Thr Cys Gly Val Pro Glu Glu Asn Lys Val Gly Ser Thr
                105                 110                 115

gaa ctt tcg cca tat cct cag gcg ttt ttg gat ggc aag agg gcc tta  480
Glu Leu Ser Pro Tyr Pro Gln Ala Phe Leu Asp Gly Lys Arg Ala Leu
            120                 125                 130

att ggg acc aac atc gtt gat tgt ggc tcg gcg ctg ctt tca agt tca  528
Ile Gly Thr Asn Ile Val Asp Cys Gly Ser Ala Leu Leu Ser Ser Ser
        135                 140                 145

gac tgt aca ccg gat aaa gtg cat atc tac cca atc cgc tgg aat gtc  576
Asp Cys Thr Pro Asp Lys Val His Ile Tyr Pro Ile Arg Trp Asn Val
    150                 155                 160

aag gca gat ggc tca ggt tcc gga ggg aat ata cga gaa ttg cgc cta  624
Lys Ala Asp Gly Ser Gly Ser Gly Gly Asn Ile Arg Glu Leu Arg Leu
165                 170                 175                 180

cat ccg gac aat gtg cac tta ggg ttc aac tcc ttc acg ttc tct aat  672
His Pro Asp Asn Val His Leu Gly Phe Asn Ser Phe Thr Phe Ser Asn
                185                 190                 195

ggt caa cta gga cag ttt ggc tac ttt agt cga ctg cag ttt aac cca  720
Gly Gln Leu Gly Gln Phe Gly Tyr Phe Ser Arg Leu Gln Phe Asn Pro
            200                 205                 210

gcc ccg aag act ggc gaa cct cgc tca gcc cga tat gat ctg gtt aac  768
Ala Pro Lys Thr Gly Glu Pro Arg Ser Ala Arg Tyr Asp Leu Val Asn
        215                 220                 225

gtt acc aga ctc tat aac ccg gac agc cca cag cct atc agc gca aaa  816
Val Thr Arg Leu Tyr Asn Pro Asp Ser Pro Gln Pro Ile Ser Ala Lys
    230                 235                 240

ggc aac gag tta ttg ttt aac cga tcg gct att gcc gtc ggt gag ctt  864
Gly Asn Glu Leu Leu Phe Asn Arg Ser Ala Ile Ala Val Gly Glu Leu
```

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245                 250                 255                 260

cga gga ttt acc gga cgt ggc aaa gaa gtc aca tat atc ggc aac cct  912
Arg Gly Phe Thr Gly Arg Gly Lys Glu Val Thr Tyr Ile Gly Asn Pro
                265                 270                 275

gtc gag tca tgc aac atc gac gta ttc gct gcc gac ctg aca acc gga  960
Val Glu Ser Cys Asn Ile Asp Val Phe Ala Ala Asp Leu Thr Thr Gly
            280                 285                 290

aag gtc cgt cgg att aca gat cac ccc gag tat gtc gat ccg atg gat  1008
Lys Val Arg Arg Ile Thr Asp His Pro Glu Tyr Val Asp Pro Met Asp
        295                 300                 305

gtc tct ccc gac gac aag tgg caa gtt atc ctc gat acc cga ggg acc  1056
Val Ser Pro Asp Asp Lys Trp Gln Val Ile Leu Asp Thr Arg Gly Thr
    310                 315                 320

ggt cga cag atg ttc atg gcc ggc atg cgc ggt att cca ccg atc atc  1104
Gly Arg Gln Met Phe Met Ala Gly Met Arg Gly Ile Pro Pro Ile Ile
325                 330                 335                 340

gac ctg ata gct act acg gtc gca tcc tct act cgc aac aac ggc cct  1152
Asp Leu Ile Ala Thr Thr Val Ala Ser Ser Thr Arg Asn Asn Gly Pro
                345                 350                 355

cgg cga ttt ttc cga cct tgg ctc ctg gac cac gat ggg gac cgt gga  1200
Arg Arg Phe Phe Arg Pro Trp Leu Leu Asp His Asp Gly Asp Arg Gly
            360                 365                 370

gac tac tat ggc cag caa atc aac ggg gac ggt gac ggc agc ccg gga  1248
Asp Tyr Tyr Gly Gln Gln Ile Asn Gly Asp Gly Asp Gly Ser Pro Gly
        375                 380                 385

agc atc aac gac cct aac tgg aac gcc ggg gca gat cca aag tgg tcc  1296
Ser Ile Asn Asp Pro Asn Trp Asn Ala Gly Ala Asp Pro Lys Trp Ser
    390                 395                 400
```

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cac gac ggc acg cgc ata gca tac ttc gag aac ctg gtt gtt tct cct   1344
His Asp Gly Thr Arg Ile Ala Tyr Phe Glu Asn Leu Val Val Ser Pro
405                 410                 415                 420

tct tgt ggc gga cag aac ccg ctg cct tgc cct aac tcc act gaa cca   1392
Ser Cys Gly Gly Gln Asn Pro Leu Pro Cys Pro Asn Ser Thr Glu Pro
                425                 430                 435

ggt ggt cgt gtc acc cgc ctg atg ctt gct cac ctg acc agc cgc gag   1440
Gly Gly Arg Val Thr Arg Leu Met Leu Ala His Leu Thr Ser Arg Glu
            440                 445                 450

ccg ctc gat ctt gaa ccc gtt gct cct gtc tct gat gaa gtt ccc tgg   1488
Pro Leu Asp Leu Glu Pro Val Ala Pro Val Ser Asp Glu Val Pro Trp
        455                 460                 465

ggt gtt cca tac gtg ccc gag agt gct cta cca gac cgt ccc ttt cca   1536
Gly Val Pro Tyr Val Pro Glu Ser Ala Leu Pro Asp Arg Pro Phe Pro
    470                 475                 480

gct gaa gga aat tac acc ttg aag gga gag gtg tca ggc tca gct tct   1584
Ala Glu Gly Asn Tyr Thr Leu Lys Gly Glu Val Ser Gly Ser Ala Ser
485                 490                 495                 500

gtg tca atc att cat gac aag acc att cca gca gcg atc aaa act atc   1632
Val Ser Ile Ile His Asp Lys Thr Ile Pro Ala Ala Ile Lys Thr Ile
                505                 510                 515

gca gtc acc tat cgc aac tat tcc gat gat ggg ttg cat gtt atc gca   1680
Ala Val Thr Tyr Arg Asn Tyr Ser Asp Asp Gly Leu His Val Ile Ala
            520                 525                 530

ggg tct gaa aga ttc acc aat act gtc gca tcc atg aca ata aac aag   1728
Gly Ser Glu Arg Phe Thr Asn Thr Val Ala Ser Met Thr Ile Asn Lys
        535                 540                 545
```

```
gtc gac tgg ttt tcc gac ctt acg tct acc gga caa gtg acc gga agc   1776
Val Asp Trp Phe Ser Asp Leu Thr Ser Thr Gly Gln Val Thr Gly Ser
    550                 555                 560

aag aag acc agt ccc ggt ggg ttc cat ctg gag att gat gct atg act   1824
Lys Lys Thr Ser Pro Gly Gly Phe His Leu Glu Ile Asp Ala Met Thr
565                 570                 575                 580

aac atc ttc atg gca aac gga acc ttg aca acc act atc gat gga aag   1872
Asn Ile Phe Met Ala Asn Gly Thr Leu Thr Thr Thr Ile Asp Gly Lys
                585                 590                 595

gtc tgg aag cag ccg gca aat ggg act tga                          1902
Val Trp Lys Gln Pro Ala Asn Gly Thr
            600                 605
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<210> 4
<211> 633
<212> PRT
<213> Aspergillus sp. PF1224

<400> 4

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Met His Val Val Ala Ser Thr Thr Ala Phe Leu Gly Val Val Ser Thr
            -25                 -20                 -15

Val Ala Gly Val His His Val Asn Arg Asp Thr Ser Gln Gln Ile Leu
        -10                 -5                  -1  1

Lys Pro Pro Val Pro Glu Pro Ile Val Val Thr Glu Leu Pro Leu Pro
  5                 10                  15                  20

Pro Val Ala Asp Ser Lys Glu Gly Ser Cys Thr Pro Glu Val Ser Pro
                25                  30                  35

His Arg Thr Gly Cys Leu Leu Lys Ser Ser Gln Ile Gln Ser Gly Asn
            40                  45                  50
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Phe Leu Pro Asp Asn Asn His Val Leu Val Ser Leu Asn Phe Ser Gly
55 60 65

Ala Pro Ala Ala Pro Asp Pro Ala Ser Ile Tyr Asn Gly Thr His Leu
70 75 80

Thr Leu Ile Lys Ala Asp Gly Thr Asn Phe Pro Ser Gly Asp Pro Trp
85 90 95 100

Lys Cys Ile Thr Cys Gly Val Pro Glu Glu Asn Lys Val Gly Ser Thr
105 110 115

Glu Leu Ser Pro Tyr Pro Gln Ala Phe Leu Asp Gly Lys Arg Ala Leu
120 125 130

Ile Gly Thr Asn Ile Val Asp Cys Gly Ser Ala Leu Leu Ser Ser Ser
135 140 145

Asp Cys Thr Pro Asp Lys Val His Ile Tyr Pro Ile Arg Trp Asn Val
150 155 160

Lys Ala Asp Gly Ser Gly Ser Gly Gly Asn Ile Arg Glu Leu Arg Leu
165 170 175 180

His Pro Asp Asn Val His Leu Gly Phe Asn Ser Phe Thr Phe Ser Asn
185 190 195

Gly Gln Leu Gly Gln Phe Gly Tyr Phe Ser Arg Leu Gln Phe Asn Pro
200 205 210

Ala Pro Lys Thr Gly Glu Pro Arg Ser Ala Arg Tyr Asp Leu Val Asn
215 220 225

Val Thr Arg Leu Tyr Asn Pro Asp Ser Pro Gln Pro Ile Ser Ala Lys
230 235 240

Gly Asn Glu Leu Leu Phe Asn Arg Ser Ala Ile Ala Val Gly Glu Leu
245 250 255 260

Arg Gly Phe Thr Gly Arg Gly Lys Glu Val Thr Tyr Ile Gly Asn Pro
265 270 275

Val Glu Ser Cys Asn Ile Asp Val Phe Ala Ala Asp Leu Thr Thr Gly
280 285 290

Lys Val Arg Arg Ile Thr Asp His Pro Glu Tyr Val Asp Pro Met Asp
295 300 305

Val Ser Pro Asp Asp Lys Trp Gln Val Ile Leu Asp Thr Arg Gly Thr
310 315 320

Gly Arg Gln Met Phe Met Ala Gly Met Arg Gly Ile Pro Pro Ile Ile
325 330 335 340

Asp Leu Ile Ala Thr Thr Val Ala Ser Ser Thr Arg Asn Asn Gly Pro
345 350 355

Arg Arg Phe Phe Arg Pro Trp Leu Leu Asp His Asp Gly Asp Arg Gly
360 365 370

Asp Tyr Tyr Gly Gln Gln Ile Asn Gly Asp Gly Asp Gly Ser Pro Gly
375 380 385

Ser Ile Asn Asp Pro Asn Trp Asn Ala Gly Ala Asp Pro Lys Trp Ser
390 395 400

His Asp Gly Thr Arg Ile Ala Tyr Phe Glu Asn Leu Val Val Ser Pro
405 410 415 420

Ser Cys Gly Gly Gln Asn Pro Leu Pro Cys Pro Asn Ser Thr Glu Pro
425 430 435

Gly Gly Arg Val Thr Arg Leu Met Leu Ala His Leu Thr Ser Arg Glu

440 445 450

Pro Leu Asp Leu Glu Pro Val Ala Pro Val Ser Asp Glu Val Pro Trp
455 460 465

Gly Val Pro Tyr Val Pro Glu Ser Ala Leu Pro Asp Arg Pro Phe Pro
470 475 480

Ala Glu Gly Asn Tyr Thr Leu Lys Gly Glu Val Ser Gly Ser Ala Ser
485 490 495 500

Val Ser Ile Ile His Asp Lys Thr Ile Pro Ala Ala Ile Lys Thr Ile
505 510 515

Ala Val Thr Tyr Arg Asn Tyr Ser Asp Asp Gly Leu His Val Ile Ala
520 525 530

Gly Ser Glu Arg Phe Thr Asn Thr Val Ala Ser Met Thr Ile Asn Lys
535 540 545

Val Asp Trp Phe Ser Asp Leu Thr Ser Thr Gly Gln Val Thr Gly Ser
550 555 560

Lys Lys Thr Ser Pro Gly Gly Phe His Leu Glu Ile Asp Ala Met Thr
565 570 575 580

Asn Ile Phe Met Ala Asn Gly Thr Leu Thr Thr Thr Ile Asp Gly Lys
585 590 595

Val Trp Lys Gln Pro Ala Asn Gly Thr
600 605

<210> 5
<211> 1857
<212> DNA
<213> Eupenicillium brefeldianum PF1226

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<222> (1)..(1854)

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<222> (52).. (1854)

<400> 5

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atg cgt tgg agt ttc tct gtt gtc cta agc acg gct gcc ctt ggt ata   48
Met Arg Trp Ser Phe Ser Val Val Leu Ser Thr Ala Ala Leu Gly Ile
        -15                 -10                 -5

tcc tcg act acc ccg gca cct cct cag cca gag ccc att gaa gta gtt   96
Ser Ser Thr Thr Pro Ala Pro Pro Gln Pro Glu Pro Ile Glu Val Val
 -1   1               5                  10                  15

gaa ctt ccc cta cct ccg gtt gca cca agc aac agt acg ggc gca tgc   144
Glu Leu Pro Leu Pro Pro Val Ala Pro Ser Asn Ser Thr Gly Ala Cys
                20                  25                  30

acg gca tcc atc aac ccc cac cgg aca ggc tgc att gcg cag gta tca   192
Thr Ala Ser Ile Asn Pro His Arg Thr Gly Cys Ile Ala Gln Val Ser
            35                  40                  45

gac tct ttc cag gct ggc gac ttt aca cct gat gga aac cat gtg gtc   240
Asp Ser Phe Gln Ala Gly Asp Phe Thr Pro Asp Gly Asn His Val Val
        50                  55                  60

atc acc gtg gag ttt gtt ggt gct ccg gcg gca cca gac cca gcc agc   288
Ile Thr Val Glu Phe Val Gly Ala Pro Ala Ala Pro Asp Pro Ala Ser
    65                  70                  75

ata tac tct ggg gaa cat atc atc ctc gtc aaa gca gac ggt aca acg   336
Ile Tyr Ser Gly Glu His Ile Ile Leu Val Lys Ala Asp Gly Thr Thr
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 80                  85                  90                  95

ttc acc aat ggc gat gca tgg aaa tgc tta agc tgc ggt gtt cct tcc  384
Phe Thr Asn Gly Asp Ala Trp Lys Cys Leu Ser Cys Gly Val Pro Ser
                100                 105                 110

aaa aat gcc ctt agc ctc gac ccg cag aga gac tat cca cat gtg gct  432
Lys Asn Ala Leu Ser Leu Asp Pro Gln Arg Asp Tyr Pro His Val Ala
            115                 120                 125

aga aat tct cga caa gcc ctt tgg gga cac aat atc ctg gat tgc agt  480
Arg Asn Ser Arg Gln Ala Leu Trp Gly His Asn Ile Leu Asp Cys Ser
        130                 135                 140

ggc att cct ctg gtc agc gat gag tgc acg cca aac aag acg cat atc  528
Gly Ile Pro Leu Val Ser Asp Glu Cys Thr Pro Asn Lys Thr His Ile
    145                 150                 155

tat cca atc tac tgg ccc acc ggc acg aac agc tcg ggc agc act cgg  576
Tyr Pro Ile Tyr Trp Pro Thr Gly Thr Asn Ser Ser Gly Ser Thr Arg
160                 165                 170                 175

gaa atg cgt ctg cat cct gac gat acg cac atg ggc tgg agc tca ttc  624
Glu Met Arg Leu His Pro Asp Asp Thr His Met Gly Trp Ser Ser Phe
                180                 185                 190

acc agt ggt ggt caa ttc gca tac ttt ggt cga ctg cag ttc cgt caa  672
Thr Ser Gly Gly Gln Phe Ala Tyr Phe Gly Arg Leu Gln Phe Arg Gln
            195                 200                 205

aat cca acc gac ggg aca ctt cgt gtt cca aga tat gat ctc gtc gat  720
Asn Pro Thr Asp Gly Thr Leu Arg Val Pro Arg Tyr Asp Leu Val Asp
        210                 215                 220

gtc aat ctg ctc gtc cag ccc aat ggt act gcg cct atc atg gcc cag  768
Val Asn Leu Leu Val Gln Pro Asn Gly Thr Ala Pro Ile Met Ala Gln
    225                 230                 235
```

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ggc tct gaa ctg aag atc cat aat gaa gct att aca gtt ggt gag ctt   816
Gly Ser Glu Leu Lys Ile His Asn Glu Ala Ile Thr Val Gly Glu Leu
240                 245                 250                 255

cgc gga ttc agc ggc gcc gga gac gag atc ctg tac att ggg tcg aca   864
Arg Gly Phe Ser Gly Ala Gly Asp Glu Ile Leu Tyr Ile Gly Ser Thr
                260                 265                 270

cgt gag gca aac aac att gat ctc ttt gcg gtc cat atc act act ggc   912
Arg Glu Ala Asn Asn Ile Asp Leu Phe Ala Val His Ile Thr Thr Gly
            275                 280                 285

gct gtt cgc cgt ctc acc agt cac cct gag tac gct gat ccc att gcc   960
Ala Val Arg Arg Leu Thr Ser His Pro Glu Tyr Ala Asp Pro Ile Ala
        290                 295                 300

ttt tca cat gac aac caa tgg ttc gtc acc atg gac act cgt ggc tca   1008
Phe Ser His Asp Asn Gln Trp Phe Val Thr Met Asp Thr Arg Gly Ser
    305                 310                 315

aat cga cag atg tgg atg gct ggg gag cgg tat att cct cct ctg att   1056
Asn Arg Gln Met Trp Met Ala Gly Glu Arg Tyr Ile Pro Pro Leu Ile
320                 325                 330                 335

gac ctg gtc act gtc aca gct gct tca tca act cgc aat aac ggc gcg   1104
Asp Leu Val Thr Val Thr Ala Ala Ser Ser Thr Arg Asn Asn Gly Ala
                340                 345                 350

cgc cgc ttc ttt cag cca atc ctg atc gat cgt tac ggt gat cgg gga   1152
Arg Arg Phe Phe Gln Pro Ile Leu Ile Asp Arg Tyr Gly Asp Arg Gly
            355                 360                 365

gac tac ttt ggt caa cga gtc aac tat caa ggc gac gga agc aat ggc   1200
Asp Tyr Phe Gly Gln Arg Val Asn Tyr Gln Gly Asp Gly Ser Asn Gly
        370                 375                 380
```

agt gtc aac gac ccg aat tgg aat ggc aga gca gac cca gcc ttc tct 1248
Ser Val Asn Asp Pro Asn Trp Asn Gly Arg Ala Asp Pro Ala Phe Ser
385 390 395

ccc gat gga act cgt atc gtc tat tgg cag gcc ttg gtg att cca cct 1296
Pro Asp Gly Thr Arg Ile Val Tyr Trp Gln Ala Leu Val Ile Pro Pro
400 405 410 415

gcc tgc ggt ggt gca aat cca ctc ccc tgc cca gtg tca act gcc caa 1344
Ala Cys Gly Gly Ala Asn Pro Leu Pro Cys Pro Val Ser Thr Ala Gln
420 425 430

gga ggt cga aca tac cga gtg atg ctg gca cgt ctt tca gat cgc aaa 1392
Gly Gly Arg Thr Tyr Arg Val Met Leu Ala Arg Leu Ser Asp Arg Lys
435 440 445

cac acg gac cca gcc cct gtt ttt gct gcg cca gat tat att tct tgg 1440
His Thr Asp Pro Ala Pro Val Phe Ala Ala Pro Asp Tyr Ile Ser Trp
450 455 460

gcg act ccg ttc cca cca ggt gca ggt ctt cct acg tct tat acc ctg 1488
Ala Thr Pro Phe Pro Pro Gly Ala Gly Leu Pro Thr Ser Tyr Thr Leu
465 470 475

cct gcg ggt aac tac act ctc tac ggc aag gct act ggg ctt gca aat 1536
Pro Ala Gly Asn Tyr Thr Leu Tyr Gly Lys Ala Thr Gly Leu Ala Asn
480 485 490 495

gcc acc ctg acc agg gac cca ctt ttc ggc agc ttt aag act gta tcc 1584
Ala Thr Leu Thr Arg Asp Pro Leu Phe Gly Ser Phe Lys Thr Val Ser
500 505 510

gtc aac tac acg aat ttc tca gat gat ggc cag cac ttt atc aat ggc 1632
Val Asn Tyr Thr Asn Phe Ser Asp Asp Gly Gln His Phe Ile Asn Gly
515 520 525

tat gaa tct gtt act ctg acg ttg tct gcc tcg aac cct tgg ctt agc 1680

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Tyr Glu Ser Val Thr Leu Thr Leu Ser Ala Ser Asn Pro Trp Leu Ser
       530                 535                 540

cat ttg gac tgg gtc tcc gat att gtg cag act ggt gct gtg aac gct  1728
His Leu Asp Trp Val Ser Asp Ile Val Gln Thr Gly Ala Val Asn Ala
    545                 550                 555

gtt aag gag act ggg tct ggt gga ttt cat ttg aca atc gat gca cag  1776
Val Lys Glu Thr Gly Ser Gly Gly Phe His Leu Thr Ile Asp Ala Gln
560                 565                 570                 575

gag aac att ttt gag gct aat ggg aca ctg act acg act gtt gat ggc  1824
Glu Asn Ile Phe Glu Ala Asn Gly Thr Leu Thr Thr Thr Val Asp Gly
                580                 585                 590

gtg acc tac cac cag ccg ctc aat ggt gca tga                     1857
Val Thr Tyr His Gln Pro Leu Asn Gly Ala
            595                 600
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<210> 6
<211> 618
<212> PRT
<213> Eupenicillium brefeldianum PF1226

<400> 6

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Met Arg Trp Ser Phe Ser Val Val Leu Ser Thr Ala Ala Leu Gly Ile
       -15                 -10                 -5

Ser Ser Thr Thr Pro Ala Pro Pro Gln Pro Glu Pro Ile Glu Val Val
 -1   1                   5                  10                  15

Glu Leu Pro Leu Pro Pro Val Ala Pro Ser Asn Ser Thr Gly Ala Cys
                20                  25                  30

Thr Ala Ser Ile Asn Pro His Arg Thr Gly Cys Ile Ala Gln Val Ser
            35                  40                  45
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Asp Ser Phe Gln Ala Gly Asp Phe Thr Pro Asp Gly Asn His Val Val
50 55 60

Ile Thr Val Glu Phe Val Gly Ala Pro Ala Ala Pro Asp Pro Ala Ser
65 70 75

Ile Tyr Ser Gly Glu His Ile Ile Leu Val Lys Ala Asp Gly Thr Thr
80 85 90 95

Phe Thr Asn Gly Asp Ala Trp Lys Cys Leu Ser Cys Gly Val Pro Ser
100 105 110

Lys Asn Ala Leu Ser Leu Asp Pro Gln Arg Asp Tyr Pro His Val Ala
115 120 125

Arg Asn Ser Arg Gln Ala Leu Trp Gly His Asn Ile Leu Asp Cys Ser
130 135 140

Gly Ile Pro Leu Val Ser Asp Glu Cys Thr Pro Asn Lys Thr His Ile
145 150 155

Tyr Pro Ile Tyr Trp Pro Thr Gly Thr Asn Ser Ser Gly Ser Thr Arg
160 165 170 175

Glu Met Arg Leu His Pro Asp Asp Thr His Met Gly Trp Ser Ser Phe
180 185 190

Thr Ser Gly Gly Gln Phe Ala Tyr Phe Gly Arg Leu Gln Phe Arg Gln
195 200 205

Asn Pro Thr Asp Gly Thr Leu Arg Val Pro Arg Tyr Asp Leu Val Asp
210 215 220

Val Asn Leu Leu Val Gln Pro Asn Gly Thr Ala Pro Ile Met Ala Gln
225 230 235

Gly Ser Glu Leu Lys Ile His Asn Glu Ala Ile Thr Val Gly Glu Leu
240 245 250 255

Arg Gly Phe Ser Gly Ala Gly Asp Glu Ile Leu Tyr Ile Gly Ser Thr
260 265 270

Arg Glu Ala Asn Asn Ile Asp Leu Phe Ala Val His Ile Thr Thr Gly
275 280 285

Ala Val Arg Arg Leu Thr Ser His Pro Glu Tyr Ala Asp Pro Ile Ala
290 295 300

Phe Ser His Asp Asn Gln Trp Phe Val Thr Met Asp Thr Arg Gly Ser
305 310 315

Asn Arg Gln Met Trp Met Ala Gly Glu Arg Tyr Ile Pro Pro Leu Ile
320 325 330 335

Asp Leu Val Thr Val Thr Ala Ala Ser Ser Thr Arg Asn Asn Gly Ala
340 345 350

Arg Arg Phe Phe Gln Pro Ile Leu Ile Asp Arg Tyr Gly Asp Arg Gly
355 360 365

Asp Tyr Phe Gly Gln Arg Val Asn Tyr Gln Gly Asp Gly Ser Asn Gly
370 375 380

Ser Val Asn Asp Pro Asn Trp Asn Gly Arg Ala Asp Pro Ala Phe Ser
385 390 395

Pro Asp Gly Thr Arg Ile Val Tyr Trp Gln Ala Leu Val Ile Pro Pro
400 405 410 415

Ala Cys Gly Gly Ala Asn Pro Leu Pro Cys Pro Val Ser Thr Ala Gln
420 425 430

Gly Gly Arg Thr Tyr Arg Val Met Leu Ala Arg Leu Ser Asp Arg Lys

435 440 445

His Thr Asp Pro Ala Pro Val Phe Ala Ala Pro Asp Tyr Ile Ser Trp
450 455 460

Ala Thr Pro Phe Pro Pro Gly Ala Gly Leu Pro Thr Ser Tyr Thr Leu
465 470 475

Pro Ala Gly Asn Tyr Thr Leu Tyr Gly Lys Ala Thr Gly Leu Ala Asn
480 485 490 495

Ala Thr Leu Thr Arg Asp Pro Leu Phe Gly Ser Phe Lys Thr Val Ser
500 505 510

Val Asn Tyr Thr Asn Phe Ser Asp Asp Gly Gln His Phe Ile Asn Gly
515 520 525

Tyr Glu Ser Val Thr Leu Thr Leu Ser Ala Ser Asn Pro Trp Leu Ser
530 535 540

His Leu Asp Trp Val Ser Asp Ile Val Gln Thr Gly Ala Val Asn Ala
545 550 555

Val Lys Glu Thr Gly Ser Gly Gly Phe His Leu Thr Ile Asp Ala Gln
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Glu Asn Ile Phe Glu Ala Asn Gly Thr Leu Thr Thr Thr Val Asp Gly
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Val Thr Tyr His Gln Pro Leu Asn Gly Ala
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Thr Leu Gln

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<400> 8

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<210> 9
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<400> 9

Trp Asn Val Ala Ala Asp Gly Ser Gly Pro Ser Gly Glu Ile Arg
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<210> 10
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<400> 10

Val Thr Ile Leu His Asn Pro Glu Gly Val Ala Pro Ile Thr Ala Lys
1 5 10 15

<210> 11
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<213> Neocosmospora vasinfecta variety vasinfecta P1225

<400> 11

Glu His Ser Asp Thr Ile Pro Trp Gly Val Pro Tyr Val Pro Gly Ser
1 5 10 15

Gln

<210> 12
<211> 13
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<400> 12

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<210> 14
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<210> 18
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<400> 20
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<210> 21
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<210> 22
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<400> 22
acagacgccg gaggagaagc g 21

<210> 23
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<400> 23
gggcatatgg cttctcctcc tgcttctg 28

<210> 24
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<400> 24
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<210> 25
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<210> 26
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<400> 26
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<400> 27

Leu Tyr Asn Pro Asp Ser Pro Gln Pro Ile Ser Ala Lys
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<210> 28
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<400> 28

Leu Gln Phe Asn Pro Ala Pro Lys
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<210> 29
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<400> 29

Val Asp Trp Phe Ser Asp Leu Thr Ser Thr Gly Gln Val Thr Gly Ser
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Lys

<210> 30
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<400> 30

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<210> 32
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<210> 34
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ggngcnggrt traaytg 17

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<400> 35
aartcngara accartc 17

<210> 36
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<400> 36
aartcrctra accartc 17

<210> 37
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<400> 37
cctcgatacc cgagggaccg 20

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gatgggttgc atgttatcgc 20

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<210> 40
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<210> 41
<211> 21
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<210> 42
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<210> 43
<211> 35
<212> DNA
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<210> 44
<211> 12
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<400> 44

Ser Thr Thr Pro Ala Pro Pro Gln Pro Glu Pro Ile
1 5 10

<210> 45
<211> 11
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<213> Eupenicillium brefeldianum PF1226

<400> 45

Ala Asp Pro Ala Phe Ser Pro Asp Gly Thr Arg
1 5 10

<210> 46
<211> 13
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<213> Eupenicillium brefeldianum PF1226

<400> 46

Leu His Pro Asp Asp Thr His Met Gly Trp Ser Ser Phe
1 5 10

<210> 47
<211> 16
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<213> Eupenicillium brefeldianum PF1226

<400> 47

Gly Phe Ser Gly Ala Gly Asp Glu Ile Leu Tyr Ile Gly Ser Thr Arg
1 5 10 15

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ccancccatr tgngtrtc 18

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<210> 52
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gggctcgagt acctcatgca ccattgagcg gctggtgg 38

<210> 53
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Met His Phe Phe Asp Lys Ala Thr Val Tyr Ala Ile Leu Cys Gly Ser
    -25                 -20                 -15

gtg gcc cag act gtt cac act gca ccc tcc gct tct cct cct gct tct   96
Val Ala Gln Thr Val His Thr Ala Pro Ser Ala Ser Pro Pro Ala Ser
-10                 -5              -1  1               5

gtt cca aac cct cct tct cct gag ccc att acc ctc aag cag cta cct   144
Val Pro Asn Pro Pro Ser Pro Glu Pro Ile Thr Leu Lys Gln Leu Pro
            10                  15                  20

ctt cct ccc atc tcc cct agc gac gac gtc ggt gct tgc acg aag cag   192
Leu Pro Pro Ile Ser Pro Ser Asp Asp Val Gly Ala Cys Thr Lys Gln
        25                  30                  35

atc aac tct cgt gga acg gga tgt ctc gcc aac ggc gtt ttt gaa acg   240
Ile Asn Ser Arg Gly Thr Gly Cys Leu Ala Asn Gly Val Phe Glu Thr
    40                  45                  50

ttt cag tct ggt gac ttt tta cct gat gga aag cat gtc atc gcc atg   288
Phe Gln Ser Gly Asp Phe Leu Pro Asp Gly Lys His Val Ile Ala Met
55                  60                  65                  70

gtc aac ttt act ggt gcg cct gct gct ccg gct gcg gga agc atc tac   336
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Val Asn Phe Thr Gly Ala Pro Ala Ala Pro Ala Ala Gly Ser Ile Tyr
                75                  80                  85

tct ggc ccg cag gtc atc atc gtc aag acg gat ggc aag aca ttt cct   384
Ser Gly Pro Gln Val Ile Ile Val Lys Thr Asp Gly Lys Thr Phe Pro
            90                  95                 100

aac gga gac ccc tgg aag tgc atc acc tgt ggt gtc cct gag aag aac   432
Asn Gly Asp Pro Trp Lys Cys Ile Thr Cys Gly Val Pro Glu Lys Asn
        105                 110                 115

gcc gtt ggt atc agc gtc aag tat gac tac ccc cag gcc ttt aag gat   480
Ala Val Gly Ile Ser Val Lys Tyr Asp Tyr Pro Gln Ala Phe Lys Asp
    120                 125                 130

ggc aaa cgt ctt ctc atc gga cac aat att ctc gac tgc ggc acc aac   528
Gly Lys Arg Leu Leu Ile Gly His Asn Ile Leu Asp Cys Gly Thr Asn
135                 140                 145                 150

cag ttg acg agc gag agc tgc aag cca gat aac acc cac atc tac cct   576
Gln Leu Thr Ser Glu Ser Cys Lys Pro Asp Asn Thr His Ile Tyr Pro
                155                 160                 165

atc cgc tgg aat gtt gct gcc gac ggt tcc ggc ccg agc ggt gaa atc   624
Ile Arg Trp Asn Val Ala Ala Asp Gly Ser Gly Pro Ser Gly Glu Ile
            170                 175                 180

cgt gag ctg cgg tta cac ccg gac aat gtc cat ctc gag ttt agc tct   672
Arg Glu Leu Arg Leu His Pro Asp Asn Val His Leu Glu Phe Ser Ser
        185                 190                 195

ttc acc ttt gct agt ggc agt att gga cag tat gcc tac ttt tct cgg   720
Phe Thr Phe Ala Ser Gly Ser Ile Gly Gln Tyr Ala Tyr Phe Ser Arg
    200                 205                 210

ctc gtt ttc aat cct tcg ccc aag act gga act ccc ctt gcg ccg cgg   768
Leu Val Phe Asn Pro Ser Pro Lys Thr Gly Thr Pro Leu Ala Pro Arg
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215                 220                 225                 230

tat gac ctg gaa aag gtt act att ctg cac aac ccc gag ggc gtt gcc   816
Tyr Asp Leu Glu Lys Val Thr Ile Leu His Asn Pro Glu Gly Val Ala
                235                 240                 245

cct atc acg gcc aag ggt aag gtt ctg tct ctg aac ccc cag gct att   864
Pro Ile Thr Ala Lys Gly Lys Val Leu Ser Leu Asn Pro Gln Ala Ile
            250                 255                 260

tcg gtt ggc gag gct cgt ggc ttc aac ggc gac gga act gag ctc act   912
Ser Val Gly Glu Ala Arg Gly Phe Asn Gly Asp Gly Thr Glu Leu Thr
        265                 270                 275

tat gtc gga agc aat att gag agc tgt aat aat gat gtc ttt gcc gtt   960
Tyr Val Gly Ser Asn Ile Glu Ser Cys Asn Asn Asp Val Phe Ala Val
    280                 285                 290

cat ctt caa act gga gtt gtt cga cgt ctt acc aac cat ccc gag tat  1008
His Leu Gln Thr Gly Val Val Arg Arg Leu Thr Asn His Pro Glu Tyr
295                 300                 305                 310

cct gac cct ctg gct ttc tcg cct gat aac aaa tgg atg gct gtc atg  1056
Pro Asp Pro Leu Ala Phe Ser Pro Asp Asn Lys Trp Met Ala Val Met
                315                 320                 325

gat acc cgc gga agt ggt cgc aac atg ttt att gcc ggc atg cga gga  1104
Asp Thr Arg Gly Ser Gly Arg Asn Met Phe Ile Ala Gly Met Arg Gly
            330                 335                 340

atc ccg ccc ctg gtt gat att gtt ggc ggt att ctg cca gcg tcg tct  1152
Ile Pro Pro Leu Val Asp Ile Val Gly Gly Ile Leu Pro Ala Ser Ser
        345                 350                 355

cgc aac aac ggt ctt cgt cgc ttc ttc cag ccg tac ctg ctt gat ttt  1200
Arg Asn Asn Gly Leu Arg Arg Phe Phe Gln Pro Tyr Leu Leu Asp Phe
    360                 365                 370
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tat ggt gac cgc ggt gac tac tac ggc caa aag ttc aac gga gat aac   1248
Tyr Gly Asp Arg Gly Asp Tyr Tyr Gly Gln Lys Phe Asn Gly Asp Asn
375                 380                 385                 390

aat ggc gtg cct ggg agt ggt gcc atc aac gat cct gag tgg aac ggt   1296
Asn Gly Val Pro Gly Ser Gly Ala Ile Asn Asp Pro Glu Trp Asn Gly
                395                 400                 405

atg gct gat ccg aga tgg tct cct gac cgc agg cag ctc gtc ttt tgg   1344
Met Ala Asp Pro Arg Trp Ser Pro Asp Arg Arg Gln Leu Val Phe Trp
            410                 415                 420

cag act cat acc gtc tcc cct tct tgt ggc ggc gcc aac cct ctc cct   1392
Gln Thr His Thr Val Ser Pro Ser Cys Gly Gly Ala Asn Pro Leu Pro
        425                 430                 435

tgc tac cct tcg aaa gag caa ggt ggc cgt aac tat cgc atg tac atc   1440
Cys Tyr Pro Ser Lys Glu Gln Gly Gly Arg Asn Tyr Arg Met Tyr Ile
    440                 445                 450

gcg acc ttt act agc cgc agc cca agc cct cct gcc ccg gtg aag gag   1488
Ala Thr Phe Thr Ser Arg Ser Pro Ser Pro Pro Ala Pro Val Lys Glu
455                 460                 465                 470

cac tcc gat acc atc ccc tgg ggc gtc ccg tac gtt ccc gga tct cag   1536
His Ser Asp Thr Ile Pro Trp Gly Val Pro Tyr Val Pro Gly Ser Gln
                475                 480                 485

gtt act cca aag cct ggc ttg gcg ggc ggt atc tac acg ctc tac ggc   1584
Val Thr Pro Lys Pro Gly Leu Ala Gly Gly Ile Tyr Thr Leu Tyr Gly
            490                 495                 500

aag gct tcg ggc gag gcc aag gtc aac atc acc tgg ggt gag gca ccc   1632
Lys Ala Ser Gly Glu Ala Lys Val Asn Ile Thr Trp Gly Glu Ala Pro
        505                 510                 515
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gag att gga acc gtc agc gtc gtg tac aag gac tat tcg ctc gac ggc   1680
Glu Ile Gly Thr Val Ser Val Val Tyr Lys Asp Tyr Ser Leu Asp Gly
    520                 525                 530

aag agc ttc ctc aac ggg aac gag agc gtc acg ggg tct gtc gag aga   1728
Lys Ser Phe Leu Asn Gly Asn Glu Ser Val Thr Gly Ser Val Glu Arg
535                 540                 545                 550

ctg act gac tat tct ttt gac tgg tat tcg gat att cgc cag acg gga   1776
Leu Thr Asp Tyr Ser Phe Asp Trp Tyr Ser Asp Ile Arg Gln Thr Gly
                555                 560                 565

gct gtc aag gga acc aag aag acg agc cct ggt gga ttc cat gct aat   1824
Ala Val Lys Gly Thr Lys Lys Thr Ser Pro Gly Gly Phe His Ala Asn
            570                 575                 580

att gat gtc atg atc aac gac ctg act tca act ggt act ctt acc acg   1872
Ile Asp Val Met Ile Asn Asp Leu Thr Ser Thr Gly Thr Leu Thr Thr
        585                 590                 595

act ctg gat ggt gtt gag tgg cgt agt cct cag agc ggc act taa       1917
Thr Leu Asp Gly Val Glu Trp Arg Ser Pro Gln Ser Gly Thr
    600                 605                 610
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<400> 54

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Val Ala Gln Thr Val His Thr Ala Pro Ser Ala Ser Pro Pro Ala Ser
-10                 -5                  -1  1               5
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Val Pro Asn Pro Pro Ser Pro Glu Pro Ile Thr Leu Lys Gln Leu Pro
10 15 20

Leu Pro Pro Ile Ser Pro Ser Asp Asp Val Gly Ala Cys Thr Lys Gln
25 30 35

Ile Asn Ser Arg Gly Thr Gly Cys Leu Ala Asn Gly Val Phe Glu Thr
40 45 50

Phe Gln Ser Gly Asp Phe Leu Pro Asp Gly Lys His Val Ile Ala Met
55 60 65 70

Val Asn Phe Thr Gly Ala Pro Ala Ala Pro Ala Ala Gly Ser Ile Tyr
75 80 85

Ser Gly Pro Gln Val Ile Ile Val Lys Thr Asp Gly Lys Thr Phe Pro
90 95 100

Asn Gly Asp Pro Trp Lys Cys Ile Thr Cys Gly Val Pro Glu Lys Asn
105 110 115

Ala Val Gly Ile Ser Val Lys Tyr Asp Tyr Pro Gln Ala Phe Lys Asp
120 125 130

Gly Lys Arg Leu Leu Ile Gly His Asn Ile Leu Asp Cys Gly Thr Asn
135 140 145 150

Gln Leu Thr Ser Glu Ser Cys Lys Pro Asp Asn Thr His Ile Tyr Pro
155 160 165

Ile Arg Trp Asn Val Ala Ala Asp Gly Ser Gly Pro Ser Gly Glu Ile
170 175 180

Arg Glu Leu Arg Leu His Pro Asp Asn Val His Leu Glu Phe Ser Ser
185 190 195

Phe Thr Phe Ala Ser Gly Ser Ile Gly Gln Tyr Ala Tyr Phe Ser Arg

200 205 210

Leu Val Phe Asn Pro Ser Pro Lys Thr Gly Thr Pro Leu Ala Pro Arg
215 220 225 230

Tyr Asp Leu Glu Lys Val Thr Ile Leu His Asn Pro Glu Gly Val Ala
235 240 245

Pro Ile Thr Ala Lys Gly Lys Val Leu Ser Leu Asn Pro Gln Ala Ile
250 255 260

Ser Val Gly Glu Ala Arg Gly Phe Asn Gly Asp Gly Thr Glu Leu Thr
265 270 275

Tyr Val Gly Ser Asn Ile Glu Ser Cys Asn Asn Asp Val Phe Ala Val
280 285 290

His Leu Gln Thr Gly Val Val Arg Arg Leu Thr Asn His Pro Glu Tyr
295 300 305 310

Pro Asp Pro Leu Ala Phe Ser Pro Asp Asn Lys Trp Met Ala Val Met
315 320 325

Asp Thr Arg Gly Ser Gly Arg Asn Met Phe Ile Ala Gly Met Arg Gly
330 335 340

Ile Pro Pro Leu Val Asp Ile Val Gly Gly Ile Leu Pro Ala Ser Ser
345 350 355

Arg Asn Asn Gly Leu Arg Arg Phe Phe Gln Pro Tyr Leu Leu Asp Phe
360 365 370

Tyr Gly Asp Arg Gly Asp Tyr Tyr Gly Gln Lys Phe Asn Gly Asp Asn
375 380 385 390

Asn Gly Val Pro Gly Ser Gly Ala Ile Asn Asp Pro Glu Trp Asn Gly
395 400 405

Met Ala Asp Pro Arg Trp Ser Pro Asp Arg Arg Gln Leu Val Phe Trp
410 415 420

Gln Thr His Thr Val Ser Pro Ser Cys Gly Gly Ala Asn Pro Leu Pro
425 430 435

Cys Tyr Pro Ser Lys Glu Gln Gly Gly Arg Asn Tyr Arg Met Tyr Ile
440 445 450

Ala Thr Phe Thr Ser Arg Ser Pro Ser Pro Pro Ala Pro Val Lys Glu
455 460 465 470

His Ser Asp Thr Ile Pro Trp Gly Val Pro Tyr Val Pro Gly Ser Gln
475 480 485

Val Thr Pro Lys Pro Gly Leu Ala Gly Gly Ile Tyr Thr Leu Tyr Gly
490 495 500

Lys Ala Ser Gly Glu Ala Lys Val Asn Ile Thr Trp Gly Glu Ala Pro
505 510 515

Glu Ile Gly Thr Val Ser Val Val Tyr Lys Asp Tyr Ser Leu Asp Gly
520 525 530

Lys Ser Phe Leu Asn Gly Asn Glu Ser Val Thr Gly Ser Val Glu Arg
535 540 545 550

Leu Thr Asp Tyr Ser Phe Asp Trp Tyr Ser Asp Ile Arg Gln Thr Gly
555 560 565

Ala Val Lys Gly Thr Lys Lys Thr Ser Pro Gly Gly Phe His Ala Asn
570 575 580

Ile Asp Val Met Ile Asn Asp Leu Thr Ser Thr Gly Thr Leu Thr Thr
585 590 595

Thr Leu Asp Gly Val Glu Trp Arg Ser Pro Gln Ser Gly Thr
600 605 610

Claims

1. A protein selected from the group consisting of:

   (a) a protein comprising an amino acid sequence selected from the group consisting of the amino acid sequences of SEQ ID NOs: 2, 4 and 6; and
   (b) a protein that has at least 95% homology to the protein comprising the amino acid sequence of the sequence described in (a) and having saponin-decomposing activity.

2. A polynucleotide encoding the protein of claim 1.

3. A polynucleotide selected from the group consisting of

   (i) a polynucleotide consisting of a DNA sequence selected from the group consisting of the DNA sequences of SEQ ID NOs: 1, 3 and 5;
   (ii) as polynucleotide that has at least 95% homology to the polynucleotide consisting of the DNA sequence of (i) and encodes a protein having saponin-decomposing activity.

4. The polynucleotide according to claim 3, wherein said (ii) consisting of a DNA sequence having at least 98% homology to the polynucleotide comprising the DNA sequence of (i).

5. The polynucleotide according to any one of claims 2 to 4, which is derived from a filamentous fungus.

6. The polynucleotide according to claim 5, wherein said filamentous fungus belongs to genus Neocosmospora, genus Eupenicillium, or genus Aspergillus.

7. The polynucleotide according to claim 6, wherein said filamentous fungus belonging to genus Neocosmospora is Neocosmospora vasinfecta var. vasinfecta PF 1225 (accession number: FERM BP-7475) or a mutant thereof.

8. The polynucleotide according to claim 6, wherein said filamentous fungus belonging to genus Eupenicillium is Eupenicillium brefeldianum PF1226 (accession number: FERM BP-7476) or a mutant thereof.

9. The polynucleotide according to claim 6, wherein said filamentous fungus belonging to genus Aspergillus is Aspergillus sp. PF1224 (accession number: FERM BP-8004) or a mutant thereof.

10. A recombinant vector comprising a polynucleotide of any one of claims 2 to 9.

11. A host transformed with the recombinant vector of claim 10.

12. The host according to claim 11, wherein the host is a microorganism.

13. The host according to claim 12, wherein the host is a filamentous fungus.

14. The host according to claim 13, wherein said filamentous fungus belongs to genus Trichoderma.

15. The host according to claim 14, wherein the host is Trichoderma viride strain MC300-1 (accession number: FERM BP-6047).

16. The host according to any one of claims 11 to 15, which expresses a saponin-decomposing enzyme.

17. A method for producing a protein of interest, which comprises culturing the host of any one of claims 11 to 16 and collecting the protein having saponin-decomposing activity from the resulting culture.

18. A method for producing soyasapogenol B, which comprises culturing the host of any one of claims 11 to 16 to produce a protein having saponin-decomposing activity, followed by using the protein to decompose a glycoside having soyasapogenol B as an aglycone.

19. A method for producing soyasagenol, B, which comprises decomposing a glycoside having soyasapogenol B as an aglycone using the protein of claim 1.

Patentansprüche

1. Protein, ausgewählt aus der Gruppe bestehend aus:

   a) einem Protein, das eine Aminosäuresequenz umfasst, die ausgewählt ist aus der Gruppe bestehend aus den Aminosäuresequenzen mit SEQ ID Nr. 2, 4 und 6; und
   b) einem Protein, das wenigstens 95% Übereinstimmung mit dem die Aminosäuresequenz mit der in a) beschriebenen Sequenz umfassenden Protein und eine saponinabbauende Wirkung hat.

2. Polynucleotid, das das Protein nach Anspruch 1 codiert.

3. Polynucleotid, ausgewählt aus der Gruppe bestehend aus:

   i) einem Polynucleotid bestehend aus einer DNA-Sequenz, die ausgewählt ist aus der Gruppe bestehend aus den DNA-Sequenzen mit SEQ ID Nr. 1, 3 und 5;
   ii) einem Protein, das wenigstens 95% Übereinstimmung mit dem aus der DNA-Sequenz i) bestehenden Polynucleotid hat und ein Protein mit saponinabbauender Wirkung codiert.

4. Polynucleotid nach Anspruch 3, wobei ii) bestehend aus einer DNA-Sequenz mit wenigstens 98% Übereinstimmung mit dem Polynucleotid, das die DNA-Sequenz von i) umfasst.

5. Polynucleotid nach einem der Ansprüche 2 bis 4, das von einem filamentösen Pilz stammt.

6. Polynucleotid nach Anspruch 5, wobei der genannte filamentöse Pilz zu Gattung Neocosmospora, Gattung Eupenicillium oder Gattung Aspergillus gehört.

7. Polynucleotid nach Anspruch 6, wobei der genannte filamentöse Pilz, der zur Gattung Neocosmospora gehört, Neocosmospora vasinfecta var. Vasinfecta PF1225 (Zugangsnummer: FERM BP-7475) oder eine Mutante davon ist.

8. Polynucleotid nach Anspruch 6, wobei der genannte filamentöse Pilz, der zur Gattung Eupenicillium gehört, Eupenicillium brefeldianum PF1226 (Zugangsnummer: FERM BP-7476) oder eine Mutante davon ist.

9. Polynucleotid nach Anspruch 6, wobei der genannte filamentöse Pilz, der zur Gattung Aspergillus gehört, Aspergillus sp. PF1224 (Zugangsnummer: FERM BP-8004) oder eine Mutante davon ist.

10. Rekombinanter Vektor, der ein Polynucleotid nach einem der Ansprüche 2 bis 9 umfasst.

11. Wirt, der mit dem rekombinanten Vektor nach Anspruch 10 transformiert ist.

12. Wirt nach Anspruch 11, wobei der Wirt ein Mikroorganismus ist.

13. Wirt nach Anspruch 12, wobei der Wirt ein filamentöser Pilz ist.

14. Wirt nach Anspruch 13, wobei der genannte filamentöse Pilz zur Gattung Trichoderma gehört.

15. Wirt nach Anspruch 14, wobei der Wirt Trichoderma viride Stamm MC300-1 (Zugangsnummer: FERM BP-6047) ist.

16. Wirt nach einem der Ansprüche 11 bis 15, der ein saponinabbauendes Enzym exprimiert.

17. Verfahren zur Herstellung eines Proteins von Interesse, das das Kultivieren des Wirts nach einem der Ansprüche 11 bis 16 und das Ernten des Proteins mit saponinabbauender Wirkung aus der resultierenden Kultur beinhaltet.

18. Verfahren zum Erzeugen von Sojasapogenol B, das Folgendes beinhaltet:

Kultivieren des Wirts nach einem der Ansprüche 11 bis 16 zum Erzeugen eines Proteins mit saponinabbauender Wirkung, gefolgt von der Verwendung des Proteins zum Abbauen eines Glycosids mit Sojasapogenol B als Aglycon.

19. Verfahren zum Erzeugen von Sojasapogenol B, das das Abbauen eines Glycosids mit Sojasapogenol B als Aglycon unter Verwendung des Proteins nach Anspruch 1 beinhaltet.

Revendications

1. Protéine sélectionnée parmi le groupe constitué de :

(a) une protéine comprenant une séquence d'acides aminés sélectionnée parmi le groupe constitué des séquences d'acides aminés de SEQ ID N° : 2, 4 et 6 ; et
(b) une protéine qui possède au moins 95 % d'homologie à la protéine comprenant la séquence d'acides aminés de la séquence décrite en (a) et ayant une activité de décomposition de saponine.

2. Polynucléotide codant pour la protéine selon la revendication 1.

3. Polynucléotide sélectionné parmi le groupe constitué de :

(i) un polynucléotide constitué d'une séquence d'ADN sélectionnée parmi le groupe constitué des séquences d'ADN de SEQ ID N° : 1, 3 et 5 ;
(ii) un polynucléotide qui possède au moins 95 % d'homologie au polynucléotide constitué de la séquence d'ADN de (i) et code pour une protéine ayant une activité de décomposition de saponine.

4. Polynucléotide selon la revendication 3, dans lequel ledit (ii) constitué d'une séquence d'ADN ayant au moins 98 % d'homologie au polynucléotide comprenant la séquence d'ADN de (i).

5. Polynucléotide selon l'une quelconque des revendications 2 à 4, qui est dérivé d'un champignon filamenteux.

6. Polynucléotide selon la revendication 5, dans lequel ledit champignon filamenteux appartient au genre Neocosmospora, genre Eupenicillium ou genre Aspergillus.

7. Polynucléotide selon la revendication 6, dans lequel ledit champignon filamenteux appartenant au genre Neocosmospora est Neocosmospora vasinfecta var. vasinfecta PF1225 (numéro d'accès : FERM BP-7475) ou un mutant de celui-ci.

8. Polynucléotide selon la revendication 6, dans lequel ledit champignon filamenteux appartenant au genre Eupenicillium est Eupenicillium brefeldianum PF1226 (numéro d'accès : FERM BP-7476) ou un mutant de celui-ci.

9. Polynucléotide selon la revendication 6, dans lequel ledit champignon filamenteux appartenant au genre Aspergillus est Aspergillus sp. PF1224 (numéro d'accès : FERM BP-8004) ou un mutant de celui-ci.

10. Vecteur recombiné comprenant un polynucléotide selon l'une quelconque des revendications 2 à 9.

11. Hôte transformé avec le vecteur recombiné selon la revendication 10.

12. Hôte selon la revendication 11, dans lequel l'hôte est un microorganisme.

13. Hôte selon la revendication 12, dans lequel l'hôte est un champignon filamenteux.

14. Hôte selon la revendication 13, dans lequel ledit champignon filamenteux appartient au genre Trichoderma.

15. Hôte selon la revendication 14, dans lequel l'hôte est Trichoderma viride souche MC300-1 (numéro d'accès : FERM BP-6047).

16. Hôte selon l'une quelconque des revendications 11 à 15, qui exprime une enzyme de décomposition de saponine.

17. Procédé de production d'une protéine pertinente, qui comprend la mise en culture de l'hôte selon l'une quelconque des revendications 11 à 16 et le recueil de la protéine ayant une activité de décomposition de saponine à partir de la culture résultante.

18. Procédé de production de soyasapogénol B, qui comprend la mise en culture de l'hôte selon l'une quelconque des revendications 11 à 16 pour produire une protéine ayant une activité de décomposition de saponine, suivie de l'utilisation de la protéine pour décomposer un glycoside ayant le soyasapogénol B comme aglycone.

19. Procédé de production de soyasapogénol B, qui comprend la décomposition d'un glycoside ayant du soyasapogénol B comme aglycone en utilisant la protéine selon la revendication 1.

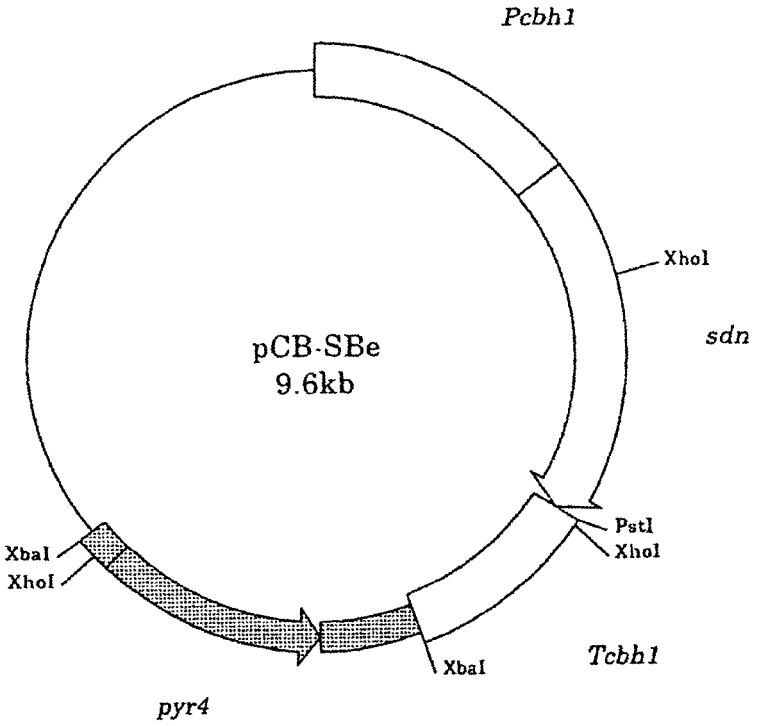
Pcbh1
XhoI
sdn
pCB-SBe
9.6kb
PstI
XhoI
XbaI
XhoI
XbaI
Tcbh1
pyr4

FIG.1

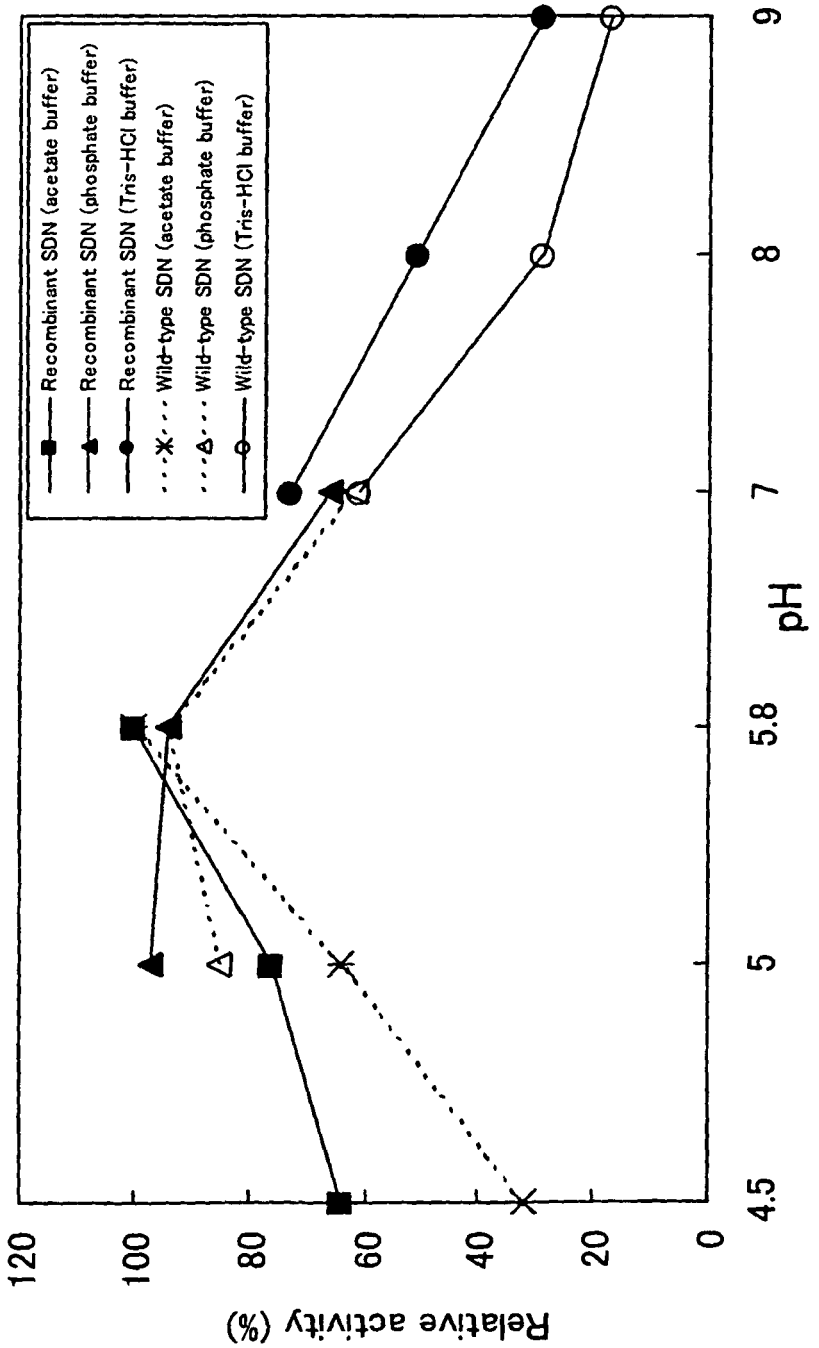
Recombinant SDN (acetate buffer)
Recombinant SDN (phosphate buffer)
Recombinant SDN (Tris-HCl buffer)
Wild-type SDN (acetate buffer)
Wild-type SDN (phosphate buffer)
Wild-type SDN (Tris-HCl buffer)
Relative activity (%)
120
100
80
60
40
20
0
4.5
5
5.8
7
8
9
pH

FIG.2

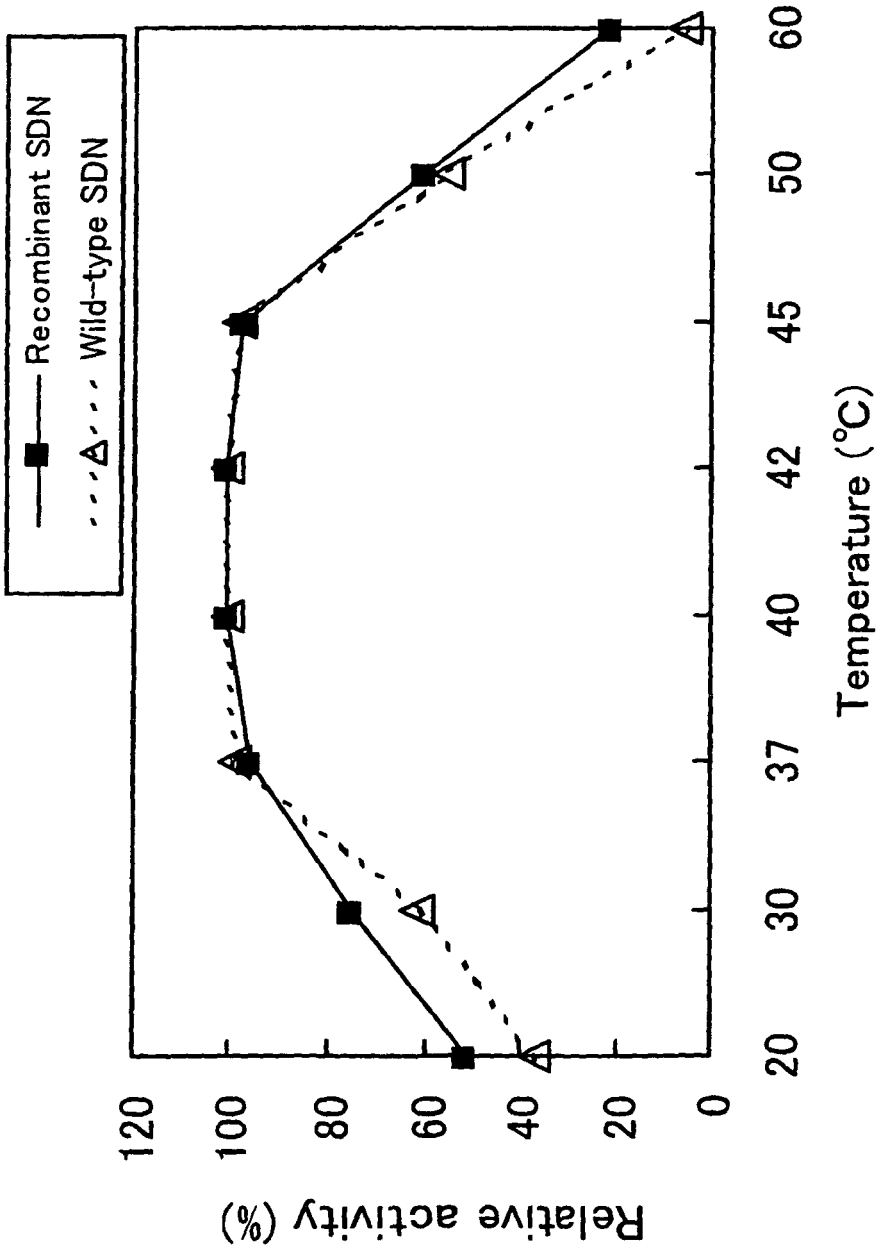
Recombinant SDN
Wild-type SDN
Relative activity (%)
120
100
80
60
40
20
0
20
30
37
40
42
45
50
60
Temperature (°C)

FIG.3

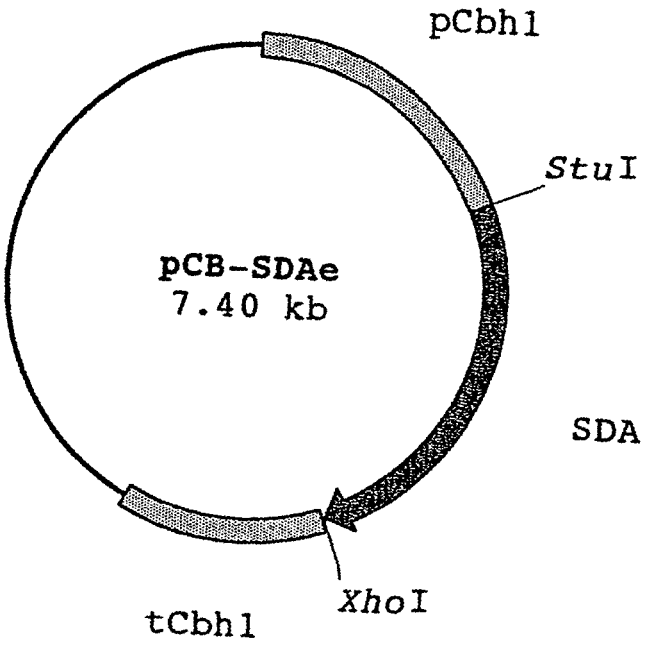

FIG.4

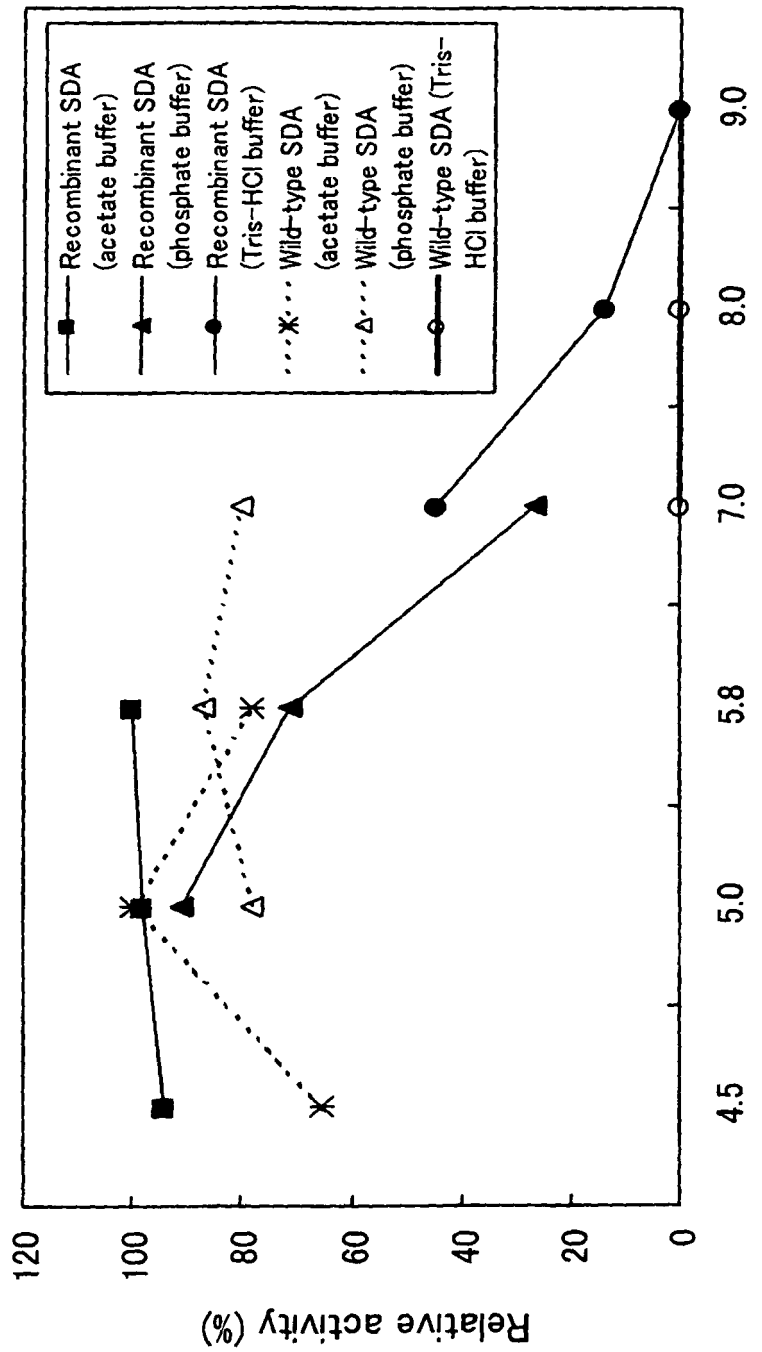
Recombinant SDA (acetate buffer)
Recombinant SDA (phosphate buffer)
Recombinant SDA (Tris-HCl buffer)
Wild-type SDA (acetate buffer)
Wild-type SDA (phosphate buffer)
Wild-type SDA (Tris-HCl buffer)
Relative activity (%)
120
100
80
60
40
20
0
4.5
5.0
5.8
7.0
8.0
9.0
pH

FIG.5

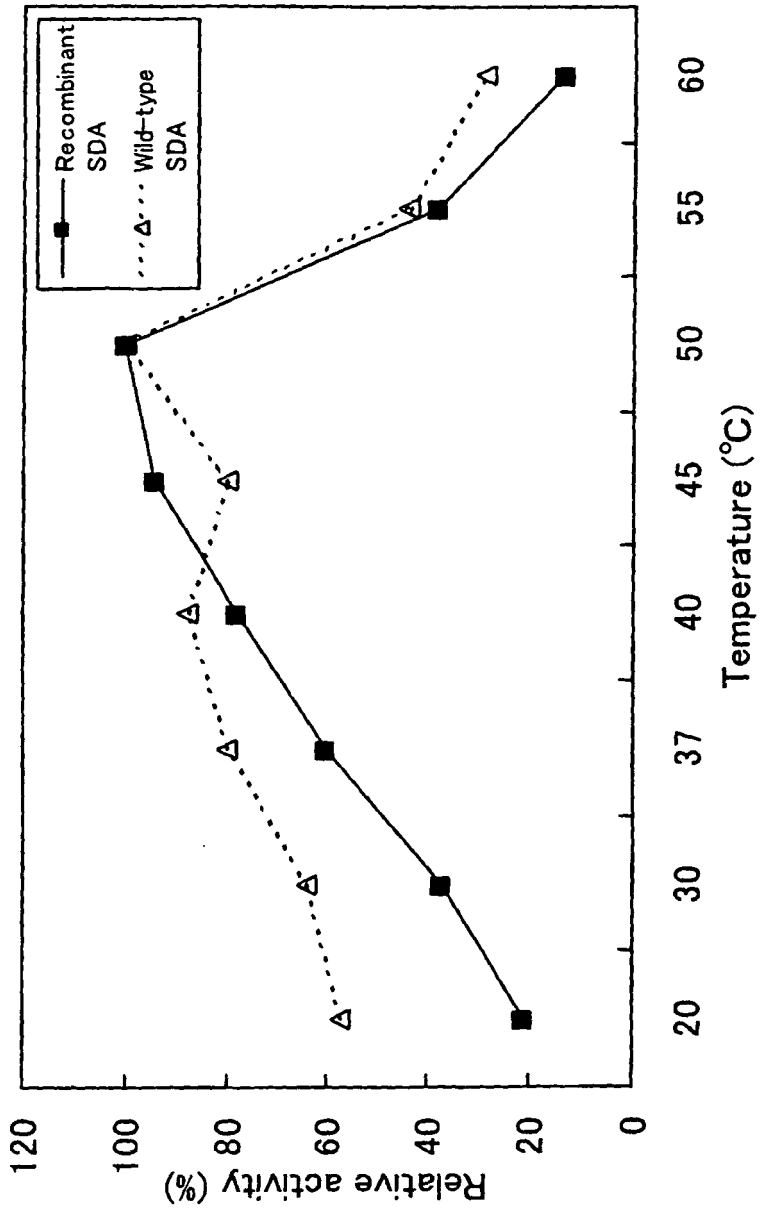
Recombinant SDA
Wild-type SDA
Relative activity (%)
120
100
80
60
40
20
0
20
30
37
40
45
50
55
60
Temperature (°C)

FIG.6

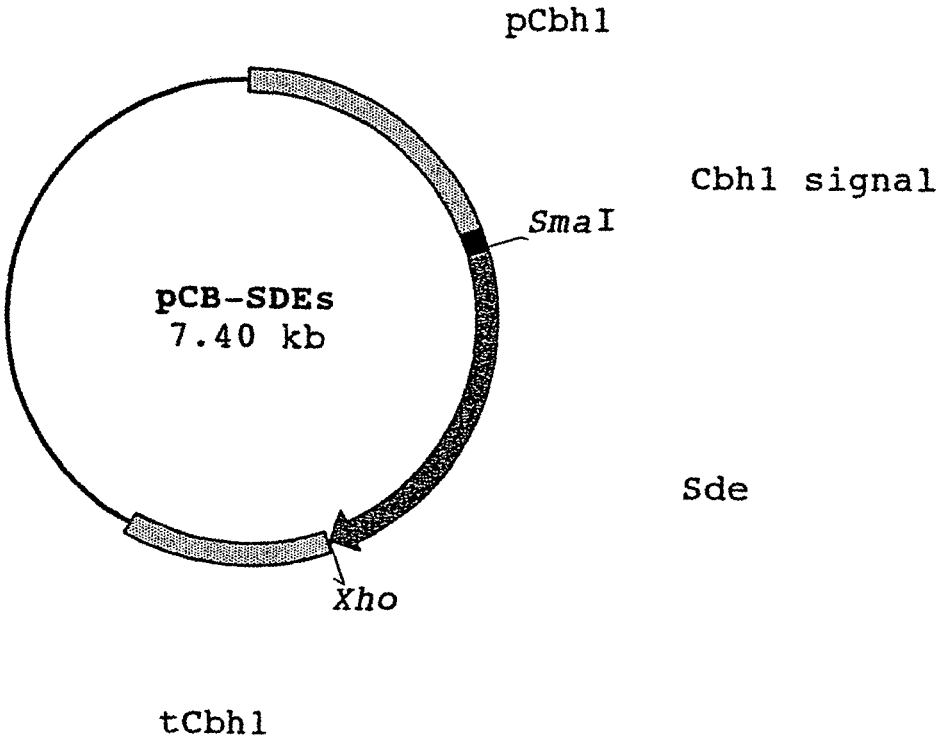

FIG.7

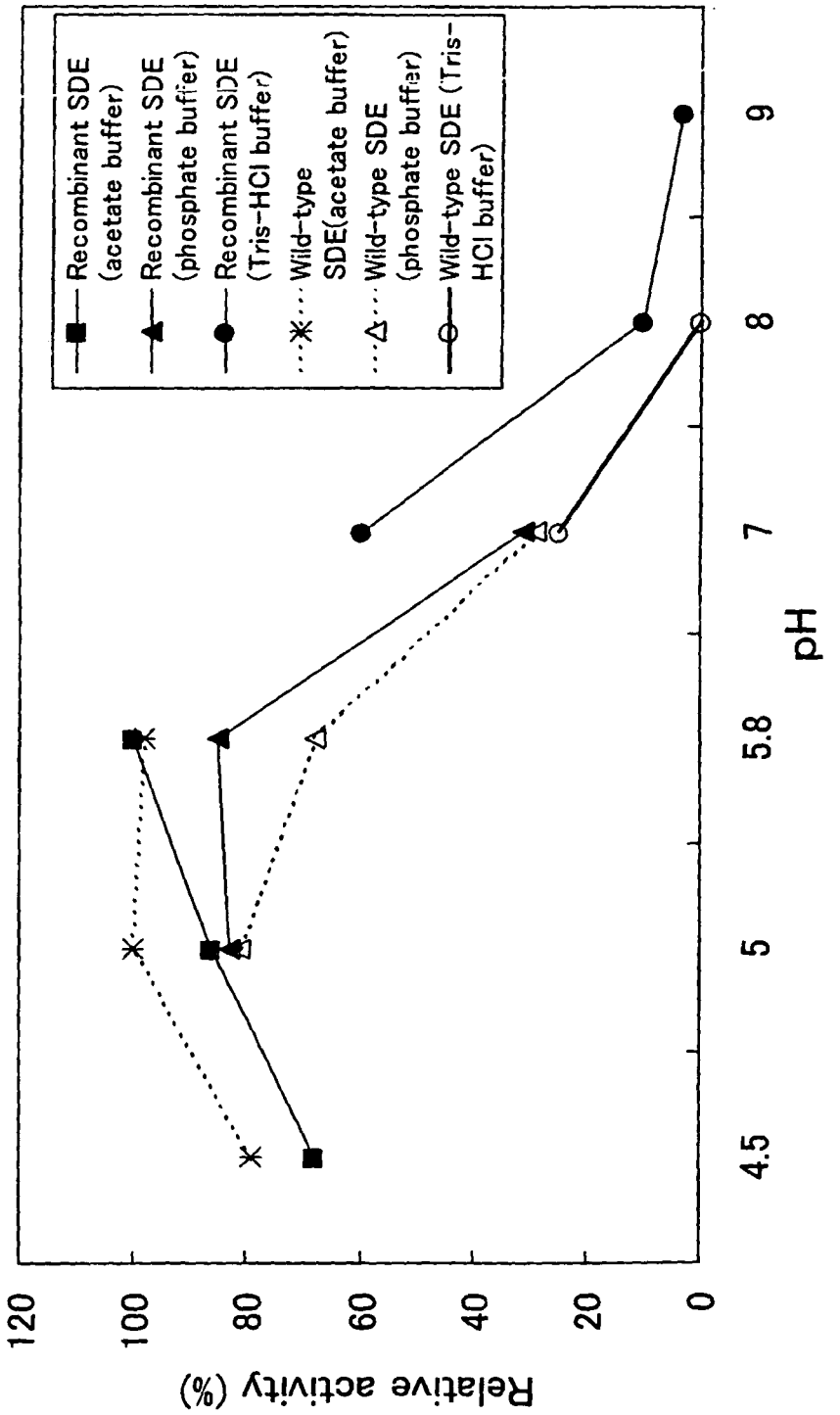
Recombinant SDE (acetate buffer)
Recombinant SDE (phosphate buffer)
Recombinant SDE (Tris-HCl buffer)
Wild-type SDE(acetate buffer)
Wild-type SDE (phosphate buffer)
Wild-type SDE (Tris-HCl buffer)
Relative activity (%)
120
100
80
60
40
20
0
4.5
5
5.8
7
8
9
pH

FIG.8

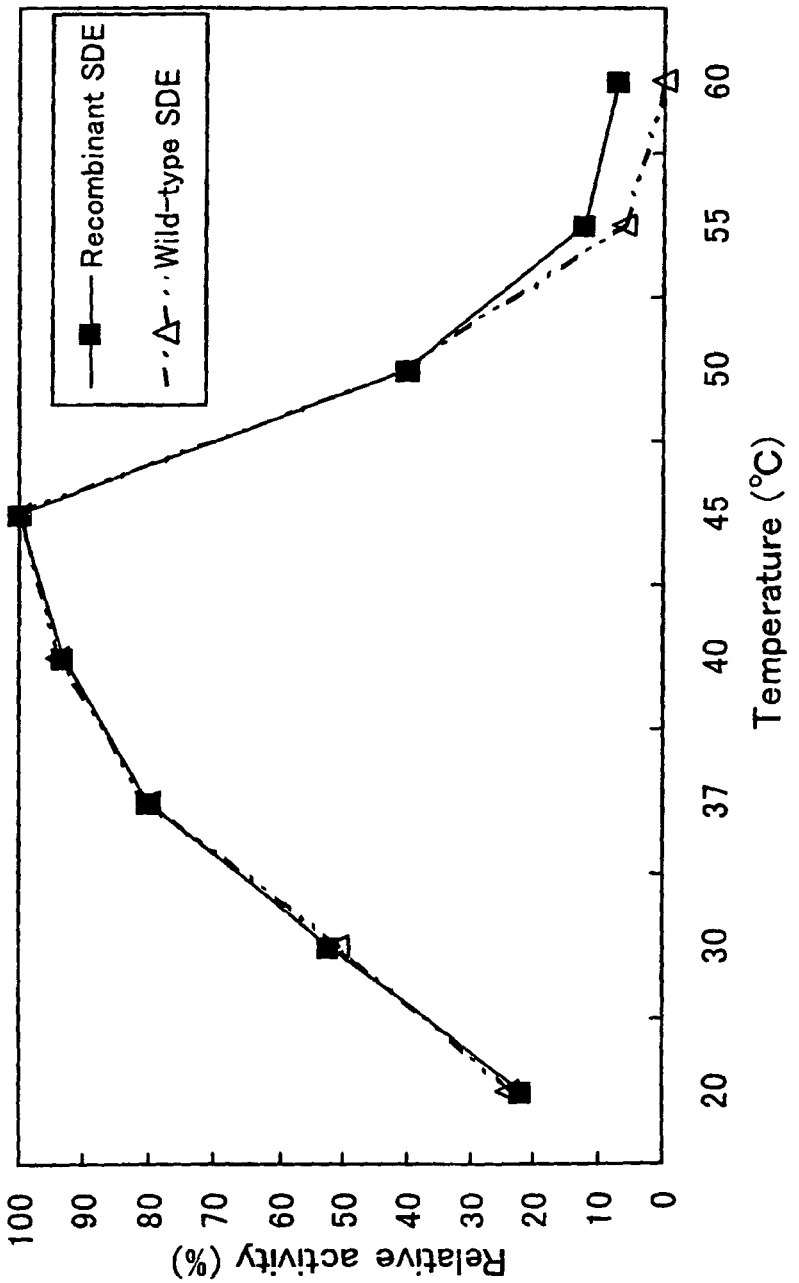
Recombinant SDE
Wild-type SDE
Relative activity (%)
0
10
20
30
40
50
60
70
80
90
100
Temperature (°C)
20
30
37
40
45
50
55
60

FIG.9

```
SDA_mat.ptn   1:QQILK---P-PVPEPIVVTELPLPPVADSKEGS-CTPEVSPHRTGCLLKSSQ--IQSGNF 53
SDE_mat.ptn   1:SII----PAPPQPEPIEVVELPLPPVAPSNSTGACTASINPHRTGCIAQVSDSFQA-GDF 55
SDN_mat.ptn   1:ASPPASVPNPPSPEPITLKQLPLPPISPSDDVGACTKQINSRGTGCLANGVFETFQSGDF 60
                      . .* **** . .*****...*   ..** ..... ***..  .    ..*.*
SDA_mat.ptn  54:LPDNNHVLVSLNFSGAPAAPDPASIYNGTHLTLIKADGTNFPSGDPWKCITCGVPEENKV 113
SDE_mat.ptn  56:TPDGNHVVITVEFVGAPAAPDPASIYSGEHIILVKADGTTFTNGDAWKCLSCGVPSKNAL 115
SDN_mat.ptn  61:LPDGKHVIAMVNFTGAPAAPAAGSIYSGPQVIIVKTDGKTFPNGDPWKCITCGVPEKNAV 120
                 .**..**   ..* ******...***.* . ...*.**..*..**.***..****..*..
SDA_mat.ptn 114:GSTELSPYPQAFLDGKRALIGTNIVDCGSALLSSSDCTPDKVHIYPIRWNVKADGSGSGG 173
SDE_mat.ptn 116:SLDPQRDYPHVARNSRQALWGHNILDCSGIPLVSDECTPNKTHIYPIYWPTGTNSSGS-- 173
SDN_mat.ptn 121:GISVKYDYPQAFKDGKRLLIGHNILDCGTNQLTSESCKPDNTHIYPIRWNVAADGSGPSG 180
                .    .**... .....*.*.**.**.   * *  *.*...*****.*..  ...**. .
SDA_mat.ptn 174:NIRELRLHPDNVHLGFNSFTFSNGQLGQFGYFSRLQFNPAPKTGEPRSARYDLVNVTRLY 233
SDE_mat.ptn 174:-TREMRLHPDDTHMGWSSFTSG----GQFAYFGRLQFRQNPTDGTLRVPRYDLVDVNLLV 228
SDN_mat.ptn 181:EIRELRLHPDNVHLEFSSFTFASGSIGQYAYFSRLVFNPSPKTGTPLAPRYDLEKVTILH 240
                 .**.*****..*.... ***.   .  **..**.**.*..  *..*...  .****.  *. *
SDA_mat.ptn 234:NPDSPQPISAKGNELLFNRSAIAVGELRGFTGRGKEVTYIGNPVESCNIDVFAADLTTGK 293
SDE_mat.ptn 229:QPNGTAPIMAQGSELKIHNEAITVGELRGFSGAGDEILYIGSTREANNIDLFAVHITTGA 288
SDN_mat.ptn 241:NPEGVAPITAKGKVLSLNPQAISVGEARGFNGDGTELTYVGSNIESCNNDVFAVHLQTGV 300
                .*. .** *.*.*  .   ** ***.*** * * * .*.*.   *..*.*.**.... **
SDA_mat.ptn 294:VRRITDHPEYVDPMDVSPDDKWQVILDTRGTGRQMFMAGMRGIPPIIDLIATTVASSTRN 353
SDE_mat.ptn 289:VRRLTSHPEYADPIAFSHDNQWFVTMDTRGSNRQMWMAGERYIPPLIDLVTVTAASSTRN 348
SDN_mat.ptn 301:VRRLTNHPEYPDPLAFSPDNKWMAVMDTRGSGRNMFIAGMRGIPPLVDIVGGILPASSRN 360
                ***.* **** ** ..*.*..* . .****..*.*..**.*.***..*..  . ..*.**
SDA_mat.ptn 354:NGPRRFFRPWLLDHDGDRGDYYGQQINGDGDGSPGS--INDPNWNAGADPKWSHDGTRIA 411
SDE_mat.ptn 349:NGARRFFQPILIDRYGDRGDYFGQRVNYQGDGSNGS--VNDPNWNGRADPAFSPDGTRIV 406
SDN_mat.ptn 361:NGLRRFFQPYLLDFYGDRGDYYGQKINGDNNGVPGSGAINDPEWNGMADPRWSPDSRQLV 420
                ** ****.* *.* . ******.** . *....*..**  .***.**.  *** . *.*.....
SDA_mat.ptn 412:YFENLVVSPSCGGQNPLPC-PNSTEPGGRVTRLMLAHLTSREPLDLEPVAPVSDEVPWGV 470
SDE_mat.ptn 407:YWQALVIPPACGGANPLPCPVSTAQG-GRTYRVMLARLSDRKHTDPAPVFAAPDYISWAT 465
SDN_mat.ptn 421:FWQTHTVSPSCGGANPLPCYPSK-EQGGRNYRMYIATFTSRSPSPPAPVKEHSDTIPWGV 479
                ... ....*.***.*****..  . .** .*..*...*. ...**   .*..*..
SDA_mat.ptn 471:PYVPESALPDRP-FPAEGNYTLKGEVSGSASVSIIHDKTIPAAIKTIAVTYRNYSDDGLH 529
SDE_mat.ptn 466:PFPPGAGLPTSYTLPA-GNYTLYGKATGLANATLTRDPLFGSFKTVS-VNYTNFSDDGQH 523
SDN_mat.ptn 480:PYVPGSQVTPKPGL-AGGIYTLYGKASGEAKVNITWGEAPEIG--TVSVVYKDYSLDGKS 536
                *..*.. ..  . ..* *.***.*...* * . . .        .  * * ..*.** .
SDA_mat.ptn 530:VIAGSERFTNTVASMTINKV--DWFSDLTSTGQVTGSKKTSPGGFHLEIDAMTNIFMANG 587
SDE_mat.ptn 524:FINGYESVTLTLSASNPWLSHLDWVSDIVQTGAVNAVKETGSGGFHLTIDAQENIFEANG 583
SDN_mat.ptn 537:FLNGNESVTGSVERLTDYSF--DWYSDIRQTGAVKGTKKTSPGGFHANIDVMINDLTSTG 594
                ...* *..* ..    .       ** **. .**.* .  *.*..****.  **..  *.  ..*
SDA_mat.ptn 588:TLTTTIDGKVWKQPANGT                                          605
SDE_mat.ptn 584:TLTTTVDGVTYHQPLNGA                                          601
SDN_mat.ptn 595:TLTTTLDGVEWRSPQSGT                                          612
                ***** **. . .* . *.
```

FIG.10

REFERENCES CITED IN THE DESCRIPTION

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